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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/18, C07K 14/475, 16/22, C12N 1/21, A61K 38/18, A01K 67/027</b>		<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/33922</b>
			<b>(43) International Publication Date:</b> 6 August 1998 (06.08.98)
<b>(21) International Application Number:</b> PCT/US98/02363		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
<b>(22) International Filing Date:</b> 2 February 1998 (02.02.98)			
<b>(30) Priority Data:</b> 08/792,019 3 February 1997 (03.02.97) US 09/016,534 30 January 1998 (30.01.98) US			
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**(54) Title:** THE NEUROTROPHIC FACTOR NNT-1

-27	1	
MDLR AGDSWGMLAC LCTVLWHLPA VPALNRTGDP GPGPSIQKTY		17
DLTRYLEHQQL RSLAGTYLNY LGPPFNEPDF NPPRLGAETL PRATVDLEVV		67
RSINDKLRLT QNYEAYSHLL CYLRGLNRQA ATAELRRSLA HFCTSLQGLL		117
GSIAGVMAAL GYPLPQPLPG TEPTWTPGPA HSDFLQKMDD FWLLKELQTW		167
	198	
LWRSAKDFNR LKKKMQPPAA AVTLHLGAHG F*		198

**(57) Abstract**

Disclosed are nucleic acids encoding novel neurotrophic factors, designated NNT-1. Also disclosed are amino acid sequences for NNT-1 polypeptides, methods for preparing NNT-1 polypeptides, and other related aspects. Such polypeptides are active in stimulating B-cell and/or T cell production, as well as reducing inflammatory responses.

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## THE NEUROTROPHIC FACTOR NNT-1

This application is a continuation-in-part of application Serial No. 08/792,019 filed February 3, 1997 which is hereby incorporated by reference.

## BACKGROUND

Field of the Invention

10 This invention relates to a novel polypeptide designated NNT-1 and related polypeptides that have neurotrophic activity, to novel nucleic acid molecules encoding such polypeptides, and to other related aspects.

15

Description of Related Art

A number of neurological disorders and diseases are caused at least in part by degeneration or death of particular classes of neurons. For example, Parkinson's disease is characterized by slowing of voluntary muscle movement, muscular rigidity, and tremor. Such symptoms are attributed at least in part to progressive degeneration of dopamine-producing neurons located in a specific region of the brain called the substantia nigra. Degeneration of these neurons ("dopaminergic neurons") results in a decrease of dopamine levels in an adjacent region of the brain called the striatum. The striatum contains neurons expressing receptors for dopamine; these neurons are involved in the control of motor activity. The cause of the degeneration of dopaminergic neurons is unknown, but has been attributed to free radicals, excess iron content, environmental toxins, excitatory amino acid neurotoxicity, and possibly a deficiency of certain neurotrophic factors (Jenner, *Neurology*, Suppl. 3:S6-

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S12 [1995]; Adams and Victor, eds. *Principles of Neurology*, Chapter 42: Degenerative Diseases of the Nervous System, McGraw Hill, NY [1993]).

Diseases such as amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease), progressive muscular atrophy, and hereditary motor and sensory neuropathy (Charcot-Marie-Tooth disease) all result at least in part from a decay of motor neurons which are located in the ventral horn of the spinal  
10 cord.

The hippocampus, a well defined structure that is part of the cerebral cortex of the brain, is important in the formation of long term memory. Destruction of the hippocampus, for example by  
15 ischemia, can result in an inability to form new memories. Degeneration of pyramidal CA1 neurons, which are located in the CA1 region of the hippocampus, is one characteristic of Alzheimer's disease. These same neurons are selectively vulnerable to ischemic and  
20 anoxic damage which occur in conditions such as stroke and head trauma. In addition, the CA1 pyramidal hippocampal neurons as well as pyramidal neurons located in the CA3 region of the hippocampus, are selectively injured in epilepsy.

25 The striatum is the innervation region of the nerve terminals of dopaminergic-containing neurons from the substantia nigra. The majority of striatal neurons utilize GABA (4-aminobutyric acid) as their neurotransmitter. The striatum is the major target of  
30 the progressive neurodegeneration that occurs in Huntington's disease, in which the major neuron loss is that of the striatal GABA-utilizing neurons.

The serotonin-containing neurons are located in groups clustered around the midline of the  
35 hindbrain. These neurons are involved in the control of body temperature, mood, and sleep. Disorders of the

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serotonin-containing neuron system include, for example, depression, other mood disorders, and sleep disturbances.

Photoreceptor cells are a specialized subset  
5 of retina neurons, and are responsible for vision. Injury and/or death of photoreceptor cells can lead to blindness. Degeneration of the retina, such as by retinitis pigmentosa, age-related macular degeneration, and stationary night blindness, are all characterized  
10 by the progressive atrophy and loss of function of photoreceptor outer segments which are specialized structures containing the visual pigments that transform a light stimulus into electrical activity.

While there are some therapies available to  
15 treat the symptoms and decrease the severity of such diseases (e.g., L-dopa to treat Parkinson's disease), there currently exists no effective treatment to prevent or reduce the degeneration of most of the above mentioned classes of affected neurons, or to promote  
20 their repair.

Recently, several naturally occurring proteinaceous molecules have been identified based on their trophic activity on various neurons. These molecules are termed "neurotrophic factors".  
25 Neurotrophic factors are endogenous, soluble proteins that can stimulate or regulate survival, growth, and/or morphological plasticity of neurons (see Fallon and Laughlin, *Neurotrophic Factors*, Academic Press, San Diego, CA [1993]).

30 The known neurotrophic factors belong to several different protein superfamilies of polypeptide growth factors based on their amino acid sequence homology and/or their three-dimensional structure (MacDonald and Hendrikson, *Cell*, 73:421-424 [1993]).  
35 One family of neurotrophic factors is the neurotrophin family. This family currently consists of NGF (nerve

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growth factor), BDNF (brain derived neurotrophic factor), NT-3 (neurotrophin-3), NT-4 (neurotrophin-4), and NT-6 (neurotrophin-6).

CNTF (ciliary neurotrophic factor) and LIF (leukemia inhibitory factor) are cytokine polypeptides that have neurotrophic activity. By virtue of their structural features and receptor components, these polypeptides are related to a family of hematopoietic cytokines that includes IL-6 (interleukin-6), IL-11 (interleukin-11), G-CSF (granulocyte-colony stimulating factor), and oncostatin-M. NNT-1 of the present invention exhibits significant similarity to various members of this family of neurotrophic factors. See FIG. 6.

GDNF (glial derived neurotrophic factor) is a neurotrophic factor that belongs to the TGF-beta (transforming growth factor beta) superfamily. GDNF displays potent survival and differentiation-promoting actions for dopaminergic and motor neurons (Lin et al., *Science*, 260:1130-1132 [1993]; Yan et al., *Nature*, 373:341-344 [1995]).

While these neurotrophic factors are known to increase growth and/or survival of neurons, there is less known about the molecules that work in conjunction with these factors. One manner in which additional neurotrophins and related molecules may be identified is to administer to an animal one or more compounds known to have an effect on the nervous system, and to then analyze tissues for the induction of genes involved in neural responses to the compounds. For example, one can screen for genes that are induced in certain tissues of the nervous system, such as the hippocampal region of the brain. This technique was used by Nedivi et al (*Nature*, 363:718-722 [1993]; Nedivi et al., *Proc. Natl. Acad. Sci USA*, 93:2048-2053 [1996]) to identify novel genes that are induced in the

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dentate gyrus portion of the hippocampus in response to administration of a neurotransmitter analog of glutamate called kainate (kainic acid).

Expression of many neurotrophic factors such as NGF, BDNF, NT3, GDNF, bFGF, IGF-1 and TGF-beta is regulated by afferent neuronal activity and/or by neuronal injury. Strong induction of some of these genes can be observed in the hippocampus dentate gyrus in response to the glutamate analog kainate (Isackson, *Current Opinions in Neurobiology* 5:50-357 [1995]). Kainate treatment appears to increase the release of novel compounds from the hippocampus of alert rats, and this activity appears to be different from the actions of known neurotrophic factors (Humpel, *et al.*, *Science*, 269:552-554 [1995]).

In view of the fact that many nervous system disorders and diseases have no known cure, there is a need in the art to identify novel compounds for treating neurological conditions and diseases such as Parkinson's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, stroke, and various degenerative disorders that affect vision.

There is additional evidence presented herein that NNT-1 compounds may have a biological activity of modulating the immune system, in particular by causing an increase in B-cell and T-cell production.

Accordingly, it is an object of the present invention to provide novel compounds that may be useful in promoting neuron regeneration and restoring neural functions.

It is a further object of the invention to provide a method of treating neurological diseases such as those set forth herein.

It is still a further object of the invention to provide a method of treating immunological diseases such as those set forth herein.

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These and other objects will be apparent to one of ordinary skill in the art from the present disclosure.

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## SUMMARY OF THE INVENTION

In one embodiment, the present invention provides a nucleic acid molecule encoding a polypeptide  
10 selected from the group consisting of:

(a) the nucleic acid molecule of SEQ ID NO:1;  
(b) the nucleic acid molecule of SEQ ID NO:3;  
(c) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:2 or a biologically active  
15 fragment thereof;

(d) a nucleic acid molecule that encodes a polypeptide that is at least 70 percent identical to the polypeptide of SEQ ID NO:2;

(e) a nucleic acid molecule that hybridizes  
20 under stringent conditions to any of (a)-(d) above; and  
(f) a nucleic acid molecule that is the complement of any of (a)-(e) above.

In another embodiment, the present invention provides a nucleic acid molecule encoding a polypeptide  
25 selected from the group consisting of:

(a') the nucleic acid molecule of SEQ ID NO:4;

(b') a nucleic acid molecule encoding the polypeptide of SEQ ID NO:5 or a biologically active  
30 fragment thereof;

(c') a nucleic acid molecule that encodes a polypeptide that is at least 70 percent identical to the polypeptide of SEQ ID NO:5;

(d') a nucleic acid molecule that hybridizes  
35 under stringent conditions to any of (a')-(c') above;  
and

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(e') a nucleic acid molecule that is the complement of any of (a')-(d') above.

In another embodiment, the invention provides vectors comprising these nucleic acid molecules, and  
5 host cells, either prokaryotic or eukaryotic, comprising the vectors.

The invention further provides an NNT-1 polypeptide selected from the group consisting of:

- (a) the polypeptide of SEQ ID NO:2;
- 10 (b) the polypeptide that is amino acids 1-198 of SEQ ID NO:2;
- (c) a polypeptide that is at least 70 percent identical to the polypeptide of (a) or (b); and
- (d) a biologically active fragment of any of  
15 (a)-(c).

The invention further provides an NNT-1 polypeptide selected from the group consisting of:

- (a') the polypeptide of SEQ ID NO:5;
- (b') the polypeptide that is amino acids 1-  
20 198 of SEQ ID NO:5;
- (c') a polypeptide that is at least 70 percent identical to the polypeptide of (a') or (b'); and
- (d') a biologically active fragment of any of  
25 (a')-(c').

Optionally, the NNT-1 polypeptide may or may not have an amino terminal methionine.

In another embodiment, the invention provides a process for producing an NNT-1 polypeptide, wherein  
30 the polypeptide may be SEQ ID NO:2 or SEQ ID NO:5, amino acids 1-198 of SEQ ID NO:2, amino acids 1-198 of SEQ ID NO: 5, or a biologically active fragment thereof, and wherein the process comprises:

- (a) expressing a polypeptide encoded by an  
35 NNT-1 nucleic acid molecule in a suitable host; and

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(b) isolating the polypeptide.  
The invention further provides anti-NNT-1  
antibodies.

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## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleic acid sequence of  
the cDNA encoding human NNT-1 (SEQ ID NO:1).

10

Figure 2 depicts the nucleic acid sequence of  
the human genomic DNA for NNT-1 (SEQ ID NO:3).

Figure 3 depicts the amino acid sequence for  
human NNT-1 (SEQ ID NO:1) as translated from the cDNA  
(SEQ ID NO:2). The first 27 amino acids may represent  
a signal peptide sequence, such that the mature form of  
NNT-1 starts at the leucine indicated as number 1. The  
\* indicates the stop codon.

20

Figure 4 depicts the nucleic acid sequence of  
the cDNA encoding murine NNT-1 (SEQ ID NO:4).

Figure 5 depicts the amino acid sequence for  
murine NNT-1 (SEQ ID NO:5) as translated from the cDNA  
(SEQ ID NO:4). The first 27 amino acids may represent  
a signal peptide sequence, such that the mature form of  
murine NNT-1 starts at the leucine indicated as number  
1. The \* indicates the stop codon.

30

Figure 6 depicts a comparison of amino acid  
sequences of NNT-1, IL-11 (SEQ ID NO:8), IL-6 (SEQ ID  
NO:9), G-CSF (SEQ ID NO:10), cardiotrophin (SEQ ID  
NO:11), CNTF (SEQ ID NO:12), oncostatin (SEQ ID NO:13),  
and LIF (SEQ ID NO:14). In each case, the human  
molecule is compared.

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Figure 7 depicts a graph of the results of a chick motor neuron activity assay for human NNT-1 compared to human CNTF.

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Figure 8 depicts a graph of the results of a chick sympathetic neuron activity assay for human NNT-1 compared to human CNTF.

10

Figure 9 depicts a normal spleen from a negative control mouse (#22), 20x objective, H&E stain.

15

Figure 10 depicts a spleen from an NNT-1 transgenic mouse (#62) with lymphoid hyperplasia (arrow).

Figure 11 depicts a normal liver from a control mouse, 10x objective, H&E stain.

20

Figure 12 depicts a liver from an NNT-1 transgenic mouse (#60) with lymphoid aggregates in sinusoids (arrow) and around vessels, H&E stain.

25

Figure 13 depicts data showing that NNT-1 induced serum SAA ( $p < 0.001$ ). There were five mice per group.

30

Figure 14 depicts data showing that NNT-1 potentiated the induction by IL-1 of corticosterone in serum ( $p < 0.01$ ) and increased serum levels of corticosterone also independently of IL-1 ( $p < 0.001$ ). There were five mice per group.

35

Figure 15 depicts data showing that NNT-1 potentiated the induction by IL-1 of IL-6 in serum ( $p < 0.001$ ). There were five mice per group.

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Figure 16 depicts data showing that NNT-1 blocked the LPS-induced increase of serum TNF levels ( $p < 0.001$ ). There were ten mice in the LPS-treated groups, five in the others.

Figure 17 depicts data showing that NNT-1 increased the counts of total ( $p < 0.04$ ) and CD45-positive cells in peripheral lymph nodes in mice ( $p < 0.001$ ).

#### DETAILED DESCRIPTION OF THE INVENTION

Included in the scope of this invention are NNT-1 polypeptides such as the polypeptides of SEQ ID NO:2 or SEQ ID NO: 5, and related biologically active polypeptide fragments and derivatives thereof. Further included within the scope of the present invention are nucleic acid molecules that encode these polypeptides, and methods for preparing the polypeptides.

##### I. NNT-1 Proteins/Polypeptides, Fragments and Derivatives Thereof

The term "NNT-1 protein" or "NNT-1 polypeptide" as used herein refers to any protein or polypeptide having the properties described herein for NNT-1. The NNT-1 polypeptide may or may not have an amino terminal methionine, depending, for example, on the manner in which it is prepared. By way of illustration, NNT-1 protein or NNT-1 polypeptide refers to:

- (1) an amino acid sequence encoded by NNT-1 nucleic acid molecules as defined in any of the following items:

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(a) the nucleic acid molecule of SEQ ID NO:1;  
(b) the nucleic acid molecule of SEQ ID NO:3;  
(c) a nucleic acid molecule encoding the  
polypeptide of SEQ ID NO:2 or a biologically active  
5 fragment thereof;

(d) a nucleic acid molecule that encodes a  
polypeptide that is at least 70 percent identical to  
the polypeptide of SEQ ID NO:2;

(e) a nucleic acid molecule that hybridizes  
10 under stringent conditions to any of (a)-(d) above; and

(f) a nucleic acid molecule that is the  
complement of any of (a)-(e) above; and

(a') the nucleic acid molecule of SEQ ID  
NO:4;

15 (b') a nucleic acid molecule encoding the  
polypeptide of SEQ ID NO:5 or a biologically active  
fragment thereof;

(c') a nucleic acid molecule that encodes a  
polypeptide that is at least 70 percent identical to  
20 the polypeptide of SEQ ID NO:5;

(d') a nucleic acid molecule that hybridizes  
under stringent conditions to any of (a')-(c') above;  
and

(e') a nucleic acid molecule that is the  
25 complement of any of (a')-(d') above; and

(2) naturally occurring allelic variants of  
the NNT-1 gene which result in one or more amino acid  
substitutions, deletions, and/or insertions as compared  
to the NNT-1 polypeptide of SEQ ID NO:2 or SEQ ID NO:  
30 5, and/or

(3) chemically modified derivatives as well  
as nucleic acid and or amino acid sequence variants  
thereof as provided for herein.

The NNT-1 polypeptides that have use in  
35 practicing the present invention may be naturally

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occurring full length polypeptides, or truncated polypeptides or peptides (*i.e.*, "fragments").

The polypeptides may be in mature form or they may be attached to a native or heterogeneous signal peptide. For example, human and murine NNT-1 have signal peptides of amino acids -27 to -1 of SEQ ID NOS: 2 and 5, respectively.

The polypeptides or fragments may be chemically modified, *i.e.*, glycosylated, phosphorylated, and/or linked to a polymer, as described below, and they may have an amino terminal methionine, depending on how they are prepared. In addition, the polypeptides or fragments may be variants of the naturally occurring NNT-1 polypeptide (*i.e.*, may contain one or more amino acid deletions, insertions, and/or substitutions as compared with naturally occurring NNT-1).

As used herein, the term "NNT-1 fragment" refers to a peptide or polypeptide that is less than the full length amino acid sequence of naturally occurring NNT-1 protein but has qualitatively a substantially similar type of biological activity as NNT-1 polypeptide or NNT-1 protein described above. Such a fragment may be truncated at the amino terminus, the carboxy terminus, or both, and may be chemically modified. Such NNT-1 fragments may be prepared with or without an amino terminal methionine. The activity of the fragments may be greater than, the same as, or less than the full-length (mature) NNT-1 polypeptide. Preferably, the activity of the fragment is  $\geq 50\%$ , more preferably  $\geq 65\%$ , most preferably  $\geq 80\%$ , of the activity of the full-length polypeptide, as measured by a standard activity assay, such as those set forth in the Examples section herein. Some exemplary fragments of this invention include the polypeptides wherein from 1 to 20 amino acids are removed from either the C-

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terminus, the N-terminus, or both termini, of the NNT-1 polypeptide.

As used herein, the term "NNT-1 derivative" or "NNT-1 variant" refers to an NNT-1 polypeptide, protein, or fragment that 1) has been chemically  
5 modified, as for example, by addition of one or more polyethylene glycol molecules, sugars, phosphates, or other such molecules not naturally attached to wild-type NNT-1 polypeptide, and/or 2) contains one or more  
10 nucleic acid or amino acid sequence substitutions, deletions, and/or insertions as compared to the NNT-1 amino acid sequence set forth in Figure 3 (human) or Figure 5 (murine).

As used herein, the terms "biologically  
15 active polypeptide" and "biologically active fragment" refer to a peptide or polypeptide in accordance with the above description for NNT-1 wherein the NNT-1 acts as a growth factor for (a) neurons (e.g., motor neurons and/or sympathetic neurons) or (b) immunological cells,  
20 such as B cells and T cells.

Fragments and/or derivatives of NNT-1 that are not themselves active in activity assays may be useful as modulators (e.g., inhibitors or stimulants) of the NNT-1 receptors *in vitro* or *in vivo*, or to  
25 prepare antibodies to NNT-1 polypeptides.

The amino acid variants of NNT-1 of this invention preferably are at least 70% identical to either SEQ ID NO: 2 or SEQ ID NO: 5, more preferably at least about 80% identical, even more preferably at  
30 least about 90% identical.

Percent sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. By way of example, using a computer  
35 program such as BLAST or FASTA, the two polypeptides for which the percent sequence identity is to be

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determined are aligned for optimal matching of their respective amino acids (the "matched span", which can include the full length of one or both sequences, or a predetermined portion of one or both sequences). Each computer program provides a "default" opening penalty and a "default" gap penalty, and a scoring matrix such as PAM 250. A standard scoring matrix (see Dayhoff et al., in: *Atlas of Protein Sequence and Structure*, vol. 5, supp.3 [1978]) can be used in conjunction with the computer program. The percent identity can then be calculated using an algorithm contained in a program such as FASTA as:

$$\frac{\text{Total number of identical matches}}{[\text{length of the longer sequence within the matched span}] + [\text{number of gaps introduced into the longer sequence in order to align the two sequences}]} \times 100$$

Polypeptides that are at least 70 percent identical will typically have one or more amino acid substitutions, deletions, and/or insertions as compared with wild type NNT-1. Usually, the substitutions will be conservative so as to have little or no effect on the overall net charge, polarity, or hydrophobicity of the protein but optionally may increase the activity of NNT-1. Conservative substitutions are set forth in Table I below.

25	<table><tr><th colspan="2">Table I</th></tr><tr><th colspan="2"><u>Conservative amino acid substitutions</u></th></tr><tr><td rowspan="3">Basic:</td><td>arginine</td></tr><tr><td>lysine</td></tr><tr><td>histidine</td></tr><tr><td rowspan="2">Acidic:</td><td>glutamic acid</td></tr><tr><td>aspartic acid</td></tr></table>	Table I		<u>Conservative amino acid substitutions</u>		Basic:	arginine	lysine	histidine	Acidic:	glutamic acid	aspartic acid
Table I												
<u>Conservative amino acid substitutions</u>												
Basic:	arginine											
	lysine											
	histidine											
Acidic:	glutamic acid											
	aspartic acid											

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Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The invention also encompasses species homologs of NNT-1; for example, NNT-1 homologs from a mammalian species such as dog, cat, mouse, rat, monkey, horse, pig, goat, rabbit, sheep and the like is contemplated in addition to human. The sequences of murine cDNA and protein are provided as SEQ ID NOS: 4 and 5.

The invention further encompasses chimeric polypeptides, such as NNT-1 attached to all or a portion of another polypeptide. Preferably the chimeric polypeptide comprises NNT-1 attached to all or a portion of another neurotrophic factor, such as BDNF, GDNF, NT-3, NT-4, NT-5, NT-6, and the like. The polypeptides may be attached N to C terminus, C to C terminus, or N to N terminus.

## II. Nucleic Acids

As used herein, the term "NNT-1" when used to describe a nucleic acid molecule refers to a nucleic acid molecule or fragment thereof, as set forth above.

The term "stringent conditions" refers to hybridization and washing under conditions that permit

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only binding of a nucleic acid molecule such as an oligonucleotide or cDNA molecule probe to highly homologous sequences. One stringent wash solution is 0.015 M NaCl, 0.005 M NaCitrate, and 0.1 percent SDS used at a temperature of 55°C-65°C. Another stringent wash solution is 0.2 X SSC and 0.1 percent SDS used at a temperature of between 50°C-65°C. Where oligonucleotide probes are used to screen cDNA or genomic libraries, the following stringent washing conditions may be used. One protocol uses 6 X SSC with 0.05 percent sodium pyrophosphate at a temperature of 35°C-62°C, depending on the length of the oligonucleotide probe. For example, 14 base pair probes are washed at 35-40°C, 17 base pair probes are washed at 45-50°C, 20 base pair probes are washed at 52-57°C, and 23 base pair probes are washed at 57-63°C. The temperature can be increased 2-3°C where the background non-specific binding appears high. A second protocol utilizes tetramethylammonium chloride (TMAC) for washing oligonucleotide probes. One stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0, and 0.2 percent SDS. The washing temperature using this solution is a function of the length of the probe. For example, a 17 base pair probe is washed at about 45-50°C.

NNT-1 nucleic acid molecules, fragments, and/or derivatives that do not themselves encode polypeptides that are active in activity assays may be useful as hybridization probes in diagnostic assays to test, either qualitatively or quantitatively, for the presence of NNT-1 DNA or RNA in mammalian tissue or bodily fluid samples.

NNT-1 nucleic acid molecules encoding NNT-1 polypeptides attached to native or heterogeneous signal peptides and/or to chimeric polypeptides as described

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herein above are also included within the scope of this invention.

### III. Methods for Preparing NNT-1 Polypeptides

#### 5 A. Recombinant Methods

The full length NNT-1 polypeptide or fragment thereof can be prepared using well known recombinant DNA technology methods such as those set forth in Sambrook et al. (*Molecular Cloning: A Laboratory*  
10 *Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1989]) and/or Ausubel et al., eds, (*Current Protocols in Molecular Biology*, Green Publishers Inc. and Wiley and Sons, NY [1994]). A gene or cDNA encoding the NNT-1 protein or fragment thereof  
15 may be obtained for example by screening a genomic or cDNA library, or by PCR amplification. Alternatively, a gene encoding the NNT-1 polypeptide or fragment may be prepared by chemical synthesis using methods well known to the skilled artisan such as those described by  
20 Engels et al. (*Angew. Chem. Intl. Ed.*, 28:716-734 [1989]). These methods include, inter alia, the phosphotriester, phosphoramidite, and H-phosphonate methods for nucleic acid synthesis. A preferred method for such chemical synthesis is polymer-supported  
25 synthesis using standard phosphoramidite chemistry. Typically, the DNA encoding the NNT-1 polypeptide will be several hundred nucleotides in length. Nucleic acids larger than about 100 nucleotides can be synthesized as several fragments using these methods.  
30 The fragments can then be ligated together to form the full length NNT-1 polypeptide. Usually, the DNA fragment encoding the amino terminus of the polypeptide will have an ATG, which encodes a methionine residue. This methionine may or may not be present on the mature  
35 form of the NNT-1 polypeptide, depending on whether the

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polypeptide produced in the host cell is secreted from that cell.

In some cases, it may be desirable to prepare nucleic acid and/or amino acid variants of naturally occurring NNT-1. Nucleic acid variants (wherein one or more nucleotides are designed to differ from the wild-type or naturally occurring NNT-1) may be produced using site directed mutagenesis or PCR amplification where the primer(s) have the desired point mutations (see Sambrook *et al.*, *supra*, and Ausubel *et al.*, *supra*, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels *et al.*, *supra*, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well. Preferred nucleic acid variants are those containing nucleotide substitutions accounting for codon preference in the host cell that is to be used to produce NNT-1. Other preferred variants are those encoding conservative amino acid changes as described above (e.g., wherein the charge or polarity of the naturally occurring amino acid side chain is not altered substantially by substitution with a different amino acid) as compared to wild type, and/or those designed to either generate a novel glycosylation and/or phosphorylation site(s) on NNT-1, or those designed to delete an existing glycosylation and/or phosphorylation site(s) on NNT-1.

The NNT-1 gene or cDNA can be inserted into an appropriate expression vector for expression in a host cell. The vector is selected to be functional in the particular host cell employed (*i.e.*, the vector is compatible with the host cell machinery such that amplification of the NNT-1 gene and/or expression of the gene can occur). The NNT-1 polypeptide or fragment thereof may be amplified/expressed in prokaryotic, yeast, insect (baculovirus systems) and/or eukaryotic

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host cells. Selection of the host cell will depend at least in part on whether the NNT-1 polypeptide or fragment thereof is to be glycosylated. If so, yeast, insect, or mammalian host cells are preferable; yeast  
5 cells will glycosylate the polypeptide, and insect and mammalian cells can glycosylate and/or phosphorylate the polypeptide as it naturally occurs on the NNT-1 polypeptide (*i.e.*, "native" glycosylation and/or phosphorylation).

10 Typically, the vectors used in any of the host cells will contain 5' flanking sequence (also referred to as a "promoter") and other regulatory elements as well such as an enhancer(s), an origin of replication element, a transcriptional termination  
15 element, a complete intron sequence containing a donor and acceptor splice site, a signal peptide sequence, a ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a  
20 selectable marker element. Each of these elements is discussed below. Optionally, the vector may contain a "tag" sequence, *i.e.*, an oligonucleotide sequence located at the 5' or 3' end of the NNT-1 coding sequence that encodes polyHis (such as hexaHis) or  
25 another small immunogenic sequence. This tag will be expressed along with the protein, and can serve as an affinity tag for purification of the NNT-1 polypeptide from the host cell. Optionally, the tag can subsequently be removed from the purified NNT-1  
30 polypeptide by various means such as using a selected peptidase for example.

The 5' flanking sequence may be homologous (*i.e.*, from the same species and/or strain as the host cell), heterologous (*i.e.*, from a species other than  
35 the host cell species or strain), hybrid (*i.e.*, a combination of 5' flanking sequences from more than one

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source), synthetic, or it may be the native NNT-1 5' flanking sequence. As such, the source of the 5' flanking sequence may be any unicellular prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the 5' flanking sequence is functional in, and can be activated by, the host cell machinery.

The 5' flanking sequences useful in the vectors of this invention may be obtained by any of several methods well known in the art. Typically, 5' flanking sequences useful herein other than the NNT-1 5' flanking sequence will have been previously identified by mapping and/or by restriction endonuclease digestion and can thus be isolated from the proper tissue source using the appropriate restriction endonucleases. In some cases, the full nucleotide sequence of the 5' flanking sequence may be known. Here, the 5' flanking sequence may be synthesized using the methods described above for nucleic acid synthesis or cloning.

Where all or only a portion of the 5' flanking sequence is known, it may be obtained using PCR and/or by screening a genomic library with suitable oligonucleotide and/or 5' flanking sequence fragments from the same or another species.

Where the 5' flanking sequence is not known, a fragment of DNA containing a 5' flanking sequence may be isolated from a larger piece of DNA that may contain, for example, a coding sequence or even another gene or genes. Isolation may be accomplished by restriction endonuclease digestion using one or more carefully selected enzymes to isolate the proper DNA fragment. After digestion, the desired fragment may be isolated by agarose gel purification, Qiagen® column or other methods known to the skilled artisan. Selection

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of suitable enzymes to accomplish this purpose will be readily apparent to one of ordinary skill in the art.

The origin of replication element is typically a part of prokaryotic expression vectors  
5 purchased commercially, and aids in the amplification of the vector in a host cell. Amplification of the vector to a certain copy number can, in some cases, be important for optimal expression of the NNT-1  
10 polypeptide. If the vector of choice does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and ligated into the vector.

The transcription termination element is typically located 3' of the end of the NNT-1  
15 polypeptide coding sequence and serves to terminate transcription of the NNT-1 polypeptide. Usually, the transcription termination element in prokaryotic cells is a G-C rich fragment followed by a poly T sequence. While the element is easily cloned from a library or  
20 even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those described above.

A selectable marker gene element encodes a protein necessary for the survival and growth of a host  
25 cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, or kanamycin for prokaryotic host cells, (b) complement auxotrophic deficiencies of  
30 the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene.

35 The ribosome binding element, commonly called the Shine-Dalgarno sequence (prokaryotes) or the Kozak

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sequence (eukaryotes), is necessary for translation initiation of mRNA. The element is typically located 3' to the promoter and 5' to the coding sequence of the NNT-1 polypeptide to be synthesized. The Shine-Dalgarno sequence is varied but is typically a polypurine (*i.e.*, having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth above and used in a prokaryotic vector.

10 In those cases where it is desirable for NNT-1 to be secreted from the host cell, a signal sequence may be used to direct the NNT-1 polypeptide out of the host cell where it is synthesized, and the carboxy-terminal part of the protein may be deleted in order to prevent membrane anchoring. Typically, the signal sequence is positioned in the coding region of NNT-1 nucleic acid sequence, or directly at the 5' end of the NNT-1 coding region. Many signal sequences have been identified, and any of them that are functional in the selected host cell may be used in conjunction with the NNT-1 gene. Therefore, the signal sequence may be homologous or heterologous to the NNT-1 polypeptide, and may be homologous or heterologous to the NNT-1 polypeptide. Additionally, the signal sequence may be chemically synthesized using methods set forth above. In most cases, secretion of the polypeptide from the host cell via the presence of a signal peptide will result in the removal of the amino terminal methionine from the polypeptide. Examples of secretory sequences useful for carrying out expression and secretion of NNT-1 polypeptides are selected from tPA leader sequences (see, *e.g.*, Rickles et al., *J. Biol. Chem.* 263: 1563-1560 [1988] and Feng et al., *J. Biol. Chem.* 265: 2022-2027 [1990], EPO leader sequences and cardiotrophin leader sequences.

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In many cases, transcription of the NNT-1 polypeptide is increased by the presence of one or more introns on the vector; this is particularly true where NNT-1 is produced in eukaryotic host cells, especially mammalian host cells. The introns used may be naturally occurring within the NNT-1 nucleic acid sequence, especially where the NNT-1 sequence used is a full length genomic sequence or a fragment thereof. Where the intron is not naturally occurring within the NNT-1 DNA sequence (as for most cDNAs), the intron(s) may be obtained from another source. The position of the intron with respect to the 5' flanking sequence and the NNT-1 coding sequence is important, as the intron must be transcribed to be effective. As such, where the NNT-1 nucleic acid sequence is a cDNA sequence, the preferred position for the intron is 3' to the transcription start site, and 5' to the polyA transcription termination sequence. Preferably for NNT-1 cDNAs, the intron will be located on one side or the other (i.e., 5' or 3') of the NNT-1 coding sequence such that it does not interrupt the this coding sequence. Any intron from any source, including any viral, prokaryotic and eukaryotic (plant or animal) organisms, may be used to practice this invention, provided that it is compatible with the host cell(s) into which it is inserted. Also included herein are synthetic introns. Optionally, more than one intron may be used in the vector.

Where one or more of the elements set forth above are not already present in the vector to be used, they may be individually obtained and ligated into the vector. Methods used for obtaining each of the elements are well known to the skilled artisan and are comparable to the methods set forth above (i.e., synthesis of the DNA, library screening, and the like).

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The final vectors used to practice this invention are typically constructed from a starting vector such as a commercially available vector. Such vectors may or may not contain some of the elements to be included in the completed vector. If none of the desired elements are present in the starting vector, each element may be individually ligated into the vector by cutting the vector with the appropriate restriction endonuclease(s) such that the ends of the element to be ligated in and the ends of the vector are compatible for ligation. In some cases, it may be necessary to "blunt" the ends to be ligated together in order to obtain a satisfactory ligation. Blunting is accomplished by first filling in "sticky ends" using Klenow DNA polymerase or T4 DNA polymerase in the presence of all four nucleotides. This procedure is well known in the art and is described for example in Sambrook et al., *supra*.

Alternatively, two or more of the elements to be inserted into the vector may first be ligated together (if they are to be positioned adjacent to each other) and then ligated into the vector.

One other method for constructing the vector is to conduct all ligations of the various elements simultaneously in one reaction mixture. Here, many nonsense or nonfunctional vectors will be generated due to improper ligation or insertion of the elements, however the functional vector may be identified and selected by restriction endonuclease digestion.

Preferred vectors for practicing this invention are those which are compatible with bacterial, insect, and/or mammalian host cells. Such vectors include, *inter alia*, pCRII (Invitrogen Company, San Diego, CA), pBSII (Stratagene Company, LaJolla, CA), and pETL (BlueBacII; Invitrogen).

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After the vector has been constructed and an NNT-1 nucleic acid has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or NNT-1 polypeptide expression.

Host cells may be prokaryotic host cells (such as *E. coli*) or eukaryotic host cells (such as a yeast cell, an insect cell, or a vertebrate cell). The host cell, when cultured under appropriate conditions, can synthesize NNT-1 protein which can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). After collection, the NNT-1 protein can be purified using methods such as molecular sieve chromatography, affinity chromatography, and the like.

Selection of the host cell will depend in part on whether the NNT-1 protein is to be glycosylated or phosphorylated (in which case eukaryotic host cells are preferred), and the manner in which the host cell is able to "fold" the protein into its native tertiary structure (e.g., proper orientation of disulfide bridges, etc.) such that biologically active protein is prepared by the cell. However, where the host cell does not synthesize biologically active NNT-1, the NNT-1 may be "folded" after synthesis using appropriate chemical conditions as discussed below.

Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7 cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines,

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including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically

5 deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

10 Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5 $\alpha$ , DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B.*

15 *subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for

20 expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention (Miller *et al.*, *Genetic Engineering* 8: 277-298 [1986]).

25 Insertion (also referred to as "transformation" or "transfection") of the vector into the selected host cell may be accomplished using such methods as calcium chloride, electroporation, microinjection, lipofection or the DEAE-dextran method.

30 The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook *et al.*, *supra*.

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The host cells containing the vector (*i.e.*, transformed or transfected) may be cultured using standard media well known to the skilled artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media for culturing *E. coli* cells are for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate, and/or fetal calf serum as necessary.

Typically, an antibiotic or other compound useful for selective growth of the transformed cells only is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin.

The amount of NNT-1 polypeptide produced in the host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, HPLC separation, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

If the NNT-1 polypeptide has been designed to be secreted from the host cells, the majority of polypeptide may be found in the cell culture medium. Polypeptides prepared in this way will typically not possess an amino terminal methionine, as it is removed during secretion from the cell. If however, the NNT-1

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polypeptide is not secreted from the host cells, it will be present in the cytoplasm (for eukaryotic, gram positive bacteria, and insect host cells) or in the periplasm (for gram negative bacteria host cells) and  
5 may have an amino terminal methionine.

For intracellular NNT-1 protein, the host cells are typically first disrupted mechanically or osmotically to release the cytoplasmic contents into a buffered solution. NNT-1 polypeptide can then be  
10 isolated from this solution.

Purification of NNT-1 polypeptide from solution can be accomplished using a variety of techniques. If the polypeptide has been synthesized such that it contains a tag such as Hexahistidine (NNT-  
15 1/hexaHis) or other small peptide at either its carboxyl or amino terminus, it may essentially be purified in a one-step process by passing the solution through an affinity column where the column matrix has a high affinity for the tag or for the polypeptide  
20 directly (i.e., a monoclonal antibody specifically recognizing NNT-1). For example, polyhistidine binds with great affinity and specificity to nickel, thus an affinity column of nickel (such as the Qiagen nickel columns) can be used for purification of NNT-1/polyHis.  
25 (See for example, Ausubel et al., eds., *Current Protocols in Molecular Biology*, Section 10.11.8, John Wiley & Sons, New York [1993]).

Where the NNT-1 polypeptide has no tag and no antibodies are available, other well known procedures  
30 for purification can be used. Such procedures include, without limitation, ion exchange chromatography, molecular sieve chromatography, HPLC, native gel electrophoresis in combination with gel elution, and preparative isoelectric focusing ("Isoprime"  
35 machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be combined to

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achieve increased purity. Preferred methods for purification include polyhistidine tagging and ion exchange chromatography in combination with preparative isoelectric focusing.

5                   If it is anticipated that the NNT-1 polypeptide will be found primarily in the periplasmic space of the bacteria or the cytoplasm of eukaryotic cells, the contents of the periplasm or cytoplasm, including inclusion bodies (e.g., gram-negative  
10 bacteria) if the processed polypeptide has formed such complexes, can be extracted from the host cell using any standard technique known to the skilled artisan. For example, the host cells can be lysed to release the contents of the periplasm by French press,  
15 homogenization, and/or sonication. The homogenate can then be centrifuged.

                  If the NNT-1 polypeptide has formed inclusion bodies in the periplasm, the inclusion bodies can often bind to the inner and/or outer cellular membranes and  
20 thus will be found primarily in the pellet material after centrifugation. The pellet material can then be treated with a chaotropic agent such as guanidine or urea to release, break apart, and solubilize the inclusion bodies. The NNT-1 polypeptide in its now  
25 soluble form can then be analyzed using gel electrophoresis, immunoprecipitation or the like. If it is desired to isolate the NNT-1 polypeptide, isolation may be accomplished using standard methods such as those set forth below and in Marston et al.  
30 (Meth. Enz., 182:264-275 [1990]).

                  If NNT-1 polypeptide inclusion bodies are not formed to a significant degree in the periplasm of the host cell, the NNT-1 polypeptide will be found primarily in the supernatant after centrifugation of  
35 the cell homogenate, and the NNT-1 polypeptide can be

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isolated from the supernatant using methods such as those set forth below.

In those situations where it is preferable to partially or completely isolate the NNT-1 polypeptide, purification can be accomplished using standard methods well known to the skilled artisan. Such methods include, without limitation, separation by electrophoresis followed by electroelution, various types of chromatography (immunoaffinity, molecular sieve, and/or ion exchange), and/or high pressure liquid chromatography. In some cases, it may be preferable to use more than one of these methods for complete purification.

15 B. Chemical Synthesis Methods

In addition to preparing and purifying NNT-1 polypeptide using recombinant DNA techniques, the NNT-1 polypeptides, fragments, and/or derivatives thereof may be prepared by chemical synthesis methods (such as solid phase peptide synthesis) using methods known in the art such as those set forth by Merrifield *et al.*, (*J. Am. Chem. Soc.*, 85:2149 [1964]), Houghten *et al.* (*Proc Natl Acad. Sci. USA*, 82:5132 [1985]), and Stewart and Young (*Solid Phase Peptide Synthesis*, Pierce Chem Co, Rockford, IL [1984]). Such polypeptides may be synthesized with or without a methionine on the amino terminus. Chemically synthesized NNT-1 polypeptides or fragments may be oxidized using methods set forth in these references to form disulfide bridges. The NNT-1 polypeptides or fragments may be employed as biologically active or immunological substitutes for natural, purified NNT-1 polypeptides in therapeutic and immunological processes.



#### IV. Chemically Modified NNT-1 Derivatives

Chemically modified NNT-1 compositions (i.e., "derivatives") where the NNT-1 polypeptide is linked to a polymer ("NNT-1-polymers") are included within the scope of the present invention. The polymer selected is typically water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer selected is usually modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so that the degree of polymerization may be controlled as provided for in the present methods. The polymer may be of any molecular weight, and may be branched or unbranched. Included within the scope of NNT-1-polymers is a mixture of polymers. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable.

The water soluble polymer or mixture thereof may be selected from the group consisting of, for example, polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol.

For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. A preferred reactive aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see U.S. Patent 5,252,714).

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Pegylation of NNT-1 may be carried out by any of the pegylation reactions known in the art, as described for example in the following references: *Focus on Growth Factors* 3: 4-10 (1992); EP 0 154 316; and EP 0 401 384. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer) as described below.

10               Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol (PEG) with an NNT-1 protein. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation of NNT-1. A preferred  
15   activated PEG ester is PEG esterified to N-hydroxysuccinimide ("NHS"). As used herein, "acylation" is contemplated to include without limitation the following types of linkages between NNT-  
20   1 and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like, as described in *Bioconjugate Chem.* 5: 133-140 (1994). Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, provided that conditions such as temperature, solvent,  
25   and pH that would inactivate the NNT-1 species to be modified are avoided.

              Pegylation by acylation usually results in a poly-pegylated NNT-1 product, wherein the lysine  $\epsilon$ -amino groups are pegylated via an acyl linking group.  
30   Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be at least about 95 percent mono, di- or tri- pegylated. However, some species with higher degrees of pegylation (up to the maximum number of lysine  $\epsilon$ -amino acid groups of  
35   NNT-1 plus one  $\alpha$ -amino group at the amino terminus of

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NNT-1) will normally be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by  
5 standard purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Pegylation by alkylation generally involves  
10 reacting a terminal aldehyde derivative of PEG with a protein such as NNT-1 in the presence of a reducing agent. Regardless of the degree of pegylation, the PEG groups are preferably attached to the protein via a -CH<sub>2</sub>-NH- group. With particular reference to the -CH<sub>2</sub>-  
15 group, this type of linkage is referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to produce a monopegylated product exploits the differential reactivity of different types of primary  
20 amino groups (lysine versus the N-terminal) available for derivatization in NNT-1. Typically, the reaction is performed at a pH (see below) which allows one to take advantage of the pK<sub>a</sub> differences between the ε-amino groups of the lysine residues and that of the  
25 α-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled: the  
30 conjugation with the polymer occurs predominantly at the N-terminus of the protein without significant modification of other reactive groups such as the lysine side chain amino groups. The present invention provides for a substantially homogeneous preparation of NNT-1-monomer polymer protein conjugate molecules (meaning  
35 NNT-1 protein to which a polymer molecule has been

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attached substantially only (i.e., at least about 95%)  
in a single location on the NNT-1 protein. More  
specifically, if polyethylene glycol is used, the  
present invention also provides for pegylated NNT-1  
5 protein lacking possibly antigenic linking groups, and  
having the polyethylene glycol molecule directly  
coupled to the NNT-1 protein.

A particularly preferred water-soluble  
polymer for use herein is polyethylene glycol,  
10 abbreviated PEG. As used herein, polyethylene glycol  
is meant to encompass any of the forms of PEG that have  
been used to derivatize other proteins, such as mono-  
(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

In general, chemical derivatization may be  
15 performed under any suitable conditions used to react a  
biologically active substance with an activated polymer  
molecule. Methods for preparing pegylated NNT-1 will  
generally comprise the steps of (a) reacting an NNT-1  
polypeptide with polyethylene glycol (such as a  
20 reactive ester or aldehyde derivative of PEG) under  
conditions whereby NNT-1 becomes attached to one or  
more PEG groups, and (b) obtaining the reaction  
product(s). In general, the optimal reaction  
conditions for the acylation reactions will be  
25 determined based on known parameters and the desired  
result. For example, the larger the ratio of PEG:  
protein, the greater the percentage of poly-pegylated  
product.

Reductive alkylation to produce a  
30 substantially homogeneous population of mono-  
polymer/NNT-1 protein conjugate molecule will generally  
comprise the steps of: (a) reacting an NNT-1 protein  
with a reactive PEG molecule under reductive alkylation  
conditions, at a pH suitable to permit selective  
35 modification of the  $\alpha$ -amino group at the amino

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terminus of said NNT-1 protein; and (b) obtaining the reaction product(s).

For a substantially homogeneous population of mono-polymer/NNT-1 protein conjugate molecules, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of NNT-1. Such reaction conditions generally provide for  $pK_a$  differences between the lysine amino groups and the  $\alpha$ -amino group at the N-terminus (the  $pK_a$  being the pH at which 50% of the amino groups are protonated and 50% are not). The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal  $\alpha$ -amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3-5, preferably 4-5.

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. In general, for the pegylation reactions contemplated herein, the preferred average molecular weight is about 2kDa to about 100kDa (the term "about" indicating  $\pm 1$ kDa). The preferred average molecular weight is about 5kDa to about 50kDa, particularly preferably about 12kDa to about 25kDa. The ratio of

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water-soluble polymer to NNT-1 protein will generally range from 1:1 to 100:1, preferably (for polypegylation) 1:1 to 20:1 and (for monopegylation) 1:1 to 5:1.

5           Using the conditions indicated above, reductive alkylation will provide for selective attachment of the polymer to any NNT-1 protein having an  $\alpha$ -amino group at the amino terminus, and provide for a substantially homogenous preparation of

10   monopolymer/NNT-1 protein conjugate. The term "monopolymer/NNT-1 protein conjugate" is used here to mean a composition comprised of a single polymer molecule attached to an NNT-1 protein molecule. The

15   monopolymer/NNT-1 protein conjugate preferably will have a polymer molecule located at the N-terminus, but not on lysine amino side groups. The preparation will preferably be greater than 90% monopolymer/NNT-1

20   protein conjugate, and more preferably greater than 95% monopolymer NNT-1 protein conjugate, with the remainder of observable molecules being unreacted (i.e., protein

25   lacking the polymer moiety). The examples below provide for a preparation which is at least about 90% monopolymer/ protein conjugate, and about 10% unreacted protein. The monopolymer/protein conjugate has

25   biological activity.

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation.

30   Preferred reducing agents may be selected from the group consisting of sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly preferred reducing agent is sodium cyanoborohydride.

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Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of products, can be determined based on the published information relating to derivatization of proteins with water soluble polymers.

A mixture of polymer-NNT-1 protein conjugate molecules may be prepared by acylation and/or alkylation methods, as described above, and one may select the proportion of monopolymer/ protein conjugate to include in the mixture. Thus, where desired, a mixture of various protein with various numbers of polymer molecules attached (i.e., di-, tri-, tetra-, etc.) may be prepared and combined with the monopolymer/NNT-1 protein conjugate material prepared using the present methods.

Generally, conditions which may be alleviated or modulated by administration of the present polymer/NNT-1 include those described herein for NNT-1 molecules in general. However, the polymer/NNT-1 molecules disclosed herein may have additional activities, enhanced or reduced activities, or other characteristics, as compared to the non-derivatized molecules.

#### V. Combinations

The NNT-1 polypeptides and fragments thereof, whether or not chemically modified, may be employed alone, or in combination with other pharmaceutical compositions such as, for example, neurotrophic factors, cytokines, interferons, interleukins, growth factors, antibiotics, anti-inflammatories, neurotransmitter receptor agonists or antagonists and/or antibodies, in the treatment of neurological or immunological system disorders.

35

VI. Antibodies

The NNT-1 polypeptides, fragments, and/or derivatives thereof may be used to prepare antibodies generated by standard methods. Thus, antibodies that  
5 react with the NNT-1 polypeptides, as well as reactive fragments of such antibodies, are also contemplated as within the scope of the present invention. The antibodies may be polyclonal, monoclonal, recombinant, chimeric, single-chain and/or bispecific. Typically,  
10 the antibody or fragment thereof will be "humanized", i.e., prepared so as to prevent or minimize an immune reaction to the antibody when administered to a patient. The antibody fragment may be any fragment that is reactive with the NNT-1 of the present  
15 invention, such as, Fab, Fab', etc. Also provided by this invention are the hybridomas generated by presenting NNT-1 or a fragment thereof as an antigen to a selected mammal, followed by fusing cells (e.g., spleen cells) of the mammal with certain cancer cells  
20 to create immortalized cell lines by known techniques. The methods employed to generate such cell lines and antibodies directed against all or portions of a human NNT-1 polypeptide of the present invention are also encompassed by this invention.

25 The antibodies may be used therapeutically, such as to inhibit binding of NNT-1 to its receptor. The antibodies may further be used for *in vivo* and *in vitro* diagnostic purposes, such as in labeled form to detect the presence of the NNT-1 in a body fluid.

30

VII. Therapeutic Compositions and Administration  
Thereof

As used herein, the terms "effective amount" and "therapeutically effective amount" refer to the



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amount of NNT-1 necessary to support one or more biological activities of NNT-1 as set forth above.

Therapeutic compositions for treating various neurological disorders or diseases are within the scope of the present invention. Such compositions may comprise a therapeutically effective amount of an NNT-1 polypeptide or fragment thereof (either of which may be chemically modified) in admixture with a pharmaceutically acceptable carrier. The carrier material may be water for injection, preferably supplemented with other materials common in solutions for administration to mammals. Typically, an NNT-1 therapeutic compound will be administered in the form of a composition comprising purified NNT-1 polypeptide or fragment (which may be chemically modified) in conjunction with one or more physiologically acceptable carriers, excipients, or diluents. Neutral buffered saline or saline mixed with serum albumin are exemplary appropriate carriers. Preferably, the product is formulated as a lyophilizate using appropriate excipients (e.g., sucrose). Other standard carriers, diluents, and excipients may be included as desired. An exemplary composition comprises citrate buffer of about pH 4.0-4.5, which may further include NaCl.

The NNT-1 compositions can be systemically administered parenterally. Alternatively, the compositions may be administered intravenously or subcutaneously. When systemically administered, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such pharmaceutically acceptable protein solutions, with due regard to pH, isotonicity, stability and the like, is within the skill of the art.

Therapeutic formulations of NNT-1 compositions useful for practicing the present

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invention may be prepared for storage by mixing the selected composition having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*Remington's*  
5 *Pharmaceutical Sciences*, 18th edition, A.R. Gennaro, ed., Mack Publishing Company [1990]) in the form of a lyophilized cake or an aqueous solution. Acceptable carriers, excipients or stabilizers are nontoxic to recipients and are preferably inert at the dosages and  
10 concentrations employed, and include buffers such as phosphate, citrate, or other organic acids; antioxidants such as ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such  
15 as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or  
20 sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

The NNT-1 composition to be used for *in vivo* administration must be sterile. This is readily  
25 accomplished by filtration through sterile filtration membranes. Where the NNT-1 composition is lyophilized, sterilization using these methods may be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral  
30 administration ordinarily will be stored in lyophilized form or in solution.

Therapeutic compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a  
35 stopper pierceable by a hypodermic injection needle.

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The route of administration of the composition is in accord with known methods, e.g. oral, injection or infusion by intravenous, intraperitoneal, intracerebral (intraparenchymal),  
5 intracerebroventricular, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems or implantation device which may optionally involve the use of a catheter. Where desired, the compositions may be administered  
10 continuously by infusion, bolus injection or by implantation device. Alternatively or additionally, NNT-1 may be administered locally via implantation into the affected area of a membrane, sponge, or other appropriate material on to which NNT-1 polypeptide has  
15 been absorbed.

Where an implantation device is used, the device may be implanted into any suitable tissue or organ, such as, for example, into a cerebral ventricle or into brain parenchyma, and delivery of NNT-1 may be  
20 directly through the device via bolus or continuous administration, or via a catheter using continuous infusion.

NNT-1 polypeptide may be administered in a sustained release formulation or preparation. Suitable  
25 examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. 3,773,919, EP 58,481), copolymers of  
30 L-glutamic acid and gamma ethyl-L-glutamine (Sidman et al, *Biopolymers*, 22: 547-556 [1983]), poly (2-hydroxyethyl-methacrylate) (Langer et al., *J. Biomed. Mater. Res.*, 15: 167-277 [1981] and Langer, *Chem. Tech.*, 12: 98-105 [1982]), ethylene vinyl acetate  
35 (Langer et al., *supra*) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release compositions also

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may include liposomes, which can be prepared by any of several methods known in the art (e.g., DE 3,218,121; Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82: 3688-3692 [1985]; Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77: 4030-4034 [1980]; EP 52,322; EP 36,676; EP 88,046; EP 143,949).

In some cases, it may be desirable to use NNT-1 compositions in an ex vivo manner, i.e., to treat cells or tissues that have been removed from the patient and are then subsequently implanted back into the patient.

In other cases, NNT-1 may be delivered through implanting into patients certain cells that have been genetically engineered to express and secrete NNT-1 polypeptide. Such cells may be animal or human cells, and may be derived from the patient's own tissue or from another source, either human or non-human. Optionally, the cells may be immortalized. The cells may be implanted into the brain, adrenal gland or into other suitable body tissues or organs of the patient.

In certain situations, it may be desirable to use gene therapy methods for administration of NNT-1 to patients suffering from certain neurological or immunological disorders. In these situations, genomic DNA, cDNA, and/or synthetic DNA encoding NNT-1 or a fragment or variant thereof may be operably linked to a constitutive or inducible promoter that is active in the tissue into which the composition will be injected. This NNT-1 DNA construct, either inserted into a vector, or alone without a vector, can be injected directly into brain or other tissue, either neuronal or non-neuronal.

Alternatively, an NNT-1 DNA construct may be directly injected into muscle tissue where it can be taken up into the cells and expressed in the cells, provided that the NNT-1 DNA is operably linked to a

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promoter that is active in muscle tissue such as cytomegalovirus (CMV) promoter, Rous sarcoma virus (RSV) promoter, or muscle creatine kinase promoter. Typically, the DNA construct may include (in addition to the NNT-1 DNA and a promoter), vector sequence  
5 obtained from vectors such as adenovirus vector, adeno-associated virus vector, a retroviral vector, and/or a herpes virus vector. The vector/DNA construct may be admixed with a pharmaceutically acceptable carrier(s)  
10 for injection.

An effective amount of the NNT-1 composition(s) to be employed therapeutically will depend, for example, upon the therapeutic objectives such as the indication for which NNT-1 is being used,  
15 the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage may range  
20 from about 0.1 µg/kg to up to 10 mg/kg or more, depending on the factors mentioned above. Typically, a clinician will administer the NNT-1 composition until a dosage is reached that achieves the desired effect. The NNT-1 composition may therefore be administered as  
25 a single dose, or as two or more doses (which may or may not contain the same amount of NNT-1) over time, or as a continuous infusion via implantation device or catheter.

As further studies are conducted, information  
30 will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, the type of disorder under treatment, the age and general health of the recipient,  
35 will be able to ascertain proper dosing.

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VIII. Conditions to be Treated with NNT-1

The NNT-1 proteins, fragments and/or derivatives thereof may be utilized to treat diseases and disorders of the central or peripheral nervous system which may be associated with alterations in the pattern of NNT-1 expression or which may benefit from exposure to NNT-1 or anti-NNT-1 antibodies.

NNT-1 protein and/or fragments or derivatives thereof, may be used to treat patients in whom various cells of the central, autonomic, or peripheral nervous system have degenerated and/or have been damaged by congenital disease, trauma, mechanical damage, surgery, stroke, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, and/or toxic agents. More specifically, NNT-1 protein levels may be modulated (up or down regulated) for such indications as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, Charcot-Marie-Tooth syndrome, Huntington's disease, peripheral neuropathy induced by diabetes or other metabolic disorder, and/or dystrophies or degeneration of the neural retina such as retinitis pigmentosa, drug-induced retinopathies, stationary forms of night blindness, progressive cone-rod degeneration, and the like. Since NNT-1 is also expressed in immune system cells (see Example V below), it may also be useful to treat diseases caused by immune disorders. Further, since NNT-1 is also expressed in hematopoietic cells (see Example V below), it may also be useful to treat diseases caused by disorders of the hematopoietic system.

In addition the NNT-1 proteins, fragments and/or derivatives thereof may be utilized to treat diseases and disorders of the immunological system involving B-cells and/or T cells, preferably B-cells. As shown in Examples IX-XI herein, NNT-1 has an

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activity of stimulating B-cell and, to a lesser degree, T-cell production.

There are several primary humoral immunodeficiencies that are potential targets for this factor. Although somewhat rare, these diseases are all chronic and would require long-term treatment. The first is common variable immunodeficiency or CVID which is characterized by somewhat normal levels of circulating B-cells but which lack the capacity to differentiate properly into immunoglobulin producing cells. Individuals with CVID are susceptible to recurrent bacterial infections.

Another NNT-1 target disease is selective IgA deficiency which also results in recurring infections, usually limited to lung, gastrointestinal and urogenital tracts. Selective IgA deficiency is one of the more common of these diseases having a prevalence between 0.03%-0.97% of the population.

Other NNT-1 target diseases include various forms of hypogammaglobulinemia, X-linked agammaglobulinemia and/or conditions related to one of these diseases such as recurring infections, renal deficiencies, or giardiasis. See, *Clin. Immunol. and Immunopath.*, 40(1):13-24 (1986).

Boosting the humoral immune response to certain vaccines may be another use for NNT-1 polypeptides. For example, antibody production following the administration of oral vaccines is often poor and therefore protects for a limited period of time. The use is envisaged of NNT-1 as an adjuvant to improve antibody production upon vaccination.

Because of its ability in inhibiting LPS-induced TNF- $\alpha$  production, NNT-1 may find use in the treatment of sepsis. Although many biological response modifier-based approaches to the solution of this very

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important clinical problem have not proved to be of any convincing validity, the possibility remains that NNT-1 may succeed there where other therapeutic candidates have failed. The Jarish-Schwarzmann reaction is a  
5 clinical condition that bears resemblances to sepsis and is strictly a consequence of TNF toxic action. The use of an anti-TNF antibody has proved to be a clinically successful approach to the treatment of this condition. This is a condition where NNT-1 may exhibit  
10 clinical value in terms of its anti-TNF and anti-inflammatory properties.

#### IX. Assays to Screen for Inhibitors of NNT-1

In some situations, it may be desirable to  
15 inhibit or significantly decrease the level of NNT-1 activity. Compounds that inhibit NNT-1 activity could be administered either in an *ex vivo* manner, or in an *in vivo* manner by local or iv injection, or by oral delivery, implantation device, or the like. The assays  
20 described below provide examples of methods useful for identifying compounds that could inhibit NNT-1 activity.

For ease of reading, the following definition is used herein for describing the assays:

25 "Test molecule(s)" refers to the molecule(s) that is under evaluation as an inhibitor of NNT-1, typically by virtue of its potential ability to block the interaction of NNT-1 with its receptor.

The NNT-1 receptor may be isolated, for  
30 example, by expression cloning using labeled (e.g., iodinated) NNT-1.

Several types of *in vitro* assays using purified protein may be conducted to identify those compounds that disrupt NNT-1 activity. Such disruption  
35 may be accomplished by a compound that typically inhibits the interaction of NNT-1 with its receptor.

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In one assay, purified NNT-1 protein or a fragment thereof (prepared for example using methods described above) can be immobilized by attachment to the bottom of the wells of a microtiter plate.

5 Radiolabeled NNT-1 receptor, as well as the test molecule(s) can then be added either one at a time or simultaneously to the wells. After incubation, the wells can be washed and counted using a scintillation counter for radioactivity to determine the degree of  
10 NNT-1/receptor binding in the presence of the test molecule. Typically, the molecule will be tested over a range of concentrations, and a series of control "wells" lacking one or more elements of the test assays can be used for accuracy in evaluating the results. A  
15 variation of this assay involves attaching the receptor to the wells, and adding radiolabeled NNT-1 along with the test molecule to the wells. After incubation and washing, the wells can be counted for radioactivity.

Several means including radiolabelling are  
20 available to "mark" NNT-1. For example, NNT-1 protein can be radiolabelled using 125-I or 35-S. Alternatively, a fusion protein of NNT-1 wherein the DNA encoding NNT-1 is fused to the coding sequence of a peptide such as the *c-myc* epitope. NNT-1-myc fusion  
25 protein can readily be detected with commercially available antibodies directed against *myc*.

An alternative to microtiter plate type of binding assays comprises immobilizing either NNT-1 or its receptor on agarose beads, acrylic beads or other  
30 types of such inert substrates. The inert substrate containing the NNT-1 or its receptor can be placed in a solution containing the test molecule along with the complementary component (either receptor or NNT-1 protein) which has been radiolabeled or fluorescently  
35 labeled; after incubation, the inert substrate can be precipitated by centrifugation, and the amount of

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binding between NNT-1 and receptor can be assessed using the methods described above. Alternatively, the insert substrate complex can be immobilized in a column and the test molecule and complementary component  
5 passed over the column. Formation of the NNT-1/receptor complex can then be assessed using any of the techniques set forth above, i.e., radiolabeling, antibody binding, or the like.

Another type of *in vitro* assay that is useful  
10 for identifying a molecule to inhibit NNT-1 activity is the Biacore assay system (Pharmacia, Piscataway, NJ) using a surface plasmon resonance detector system and following the manufacturer's protocol. This assay essentially involves covalent binding of either NNT-1  
15 or its receptor to a dextran-coated sensor chip which is located in a detector. The test molecule and the complementary component can then be injected into the chamber containing the sensor chip either simultaneously or sequentially, and the amount of  
20 binding of NNT-1/receptor can be assessed based on the change in molecular mass which is physically associated with the dextran-coated side of the of the sensor chip; the change in molecular mass can be measured by the detector system.

25 In some cases, it may be desirable to evaluate two or more test molecules together for use in decreasing or inhibiting NNT-1 activity. In these cases, the assays set forth above can be readily modified by adding such additional test molecule(s)  
30 either simultaneously with, or subsequently to, the first test molecule. The remainder of steps in the assay can be as set forth above.

#### X. Transgenic Mammals

35 Also included within the scope of the present invention are non-human mammals such as mice, rats,

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rabbits, goats, or sheep in which the gene (or genes) encoding the human equivalent of NNT-1 has been disrupted ("knocked out") such that the level of expression of this gene is significantly decreased or  
5 completely abolished. Such mammals may be prepared using techniques and methods such as those described in U.S. Patent No. 5,557,032. The present invention further includes non-human mammals such as mice, rats, rabbits, goats, or sheep in which the gene (or genes)  
10 encoding the NNT-1 (either the native form of NNT-1 for the mammal or a heterologous NNT-1 gene) is over expressed by the mammal, thereby creating a "transgenic" mammal. Such transgenic mammals may be prepared using well known methods such as those  
15 described in U.S. Patent No 5,489,743 and PCT patent application no. WO94/28122, published 8 December 1994.

The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the invention in any way.

20

## EXAMPLES

Standard methods for library preparation, DNA cloning, and protein expression are set forth in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold  
25 Spring Harbor, NY [1989]) and in Ausubel *et al.*, eds. (*Current Protocols in Molecular Biology*, Wiley, New York, NY [1995]).

Example I: Cloning of cDNA and Genomic Clone for NNT-1

## A. Construction of cDNA library

5 Human T-cell lymphoma cells, Jurkat cells, were grown at 37°C under 5% CO<sub>2</sub> in a RPMI 400 media containing 10% fetal bovine serum. The media was buffered with 10mM HEPES, pH 7.5. After 8 passages the cells were divided into two groups. One group was  
10 grown to confluency (2x10<sup>7</sup> cells/flask), the RNA harvested from these cells served as the "driver" RNA. The other group was the "tester" group and were activated with the following treatment.

The cells were activated for 8 hours by  
15 adding the superantigens Streptococci enterotoxin B and F(TSST) 80 ng/ml; the PKC activator, PMA 50 ng/ml; calcium ionophore A21832 125 ng/ml. The protein translation inhibitor cycloheximide was also added at a concentration of 1 mg/ml. RNA was harvested from the  
20 different groups of cells at different time points.

## 1. Total RNA preparation:

The cells were pelleted by centrifugation at 300 xg for 5 min and washed with PBS (phosphate  
25 buffered saline), and resuspended in Ultraspec II (Biotex, Inc., TX), at a concentration of 5x10<sup>6</sup> cells/ml of Ultraspec II. The cells were then lysed by four passages through a 21-gauge syringe. The homogenate was incubated on ice for 15 min, 0.2 volumes  
30 of chloroform was then added, mixed well, and reincubated on ice for a further 10 min, centrifuged at 12000 xg for 30 min in 30 ml corex tubes. Post-centrifugation and supernatant was saved and the residue discarded. 0.05 volumes of the RNA binding  
35 resin sold by Biotex as part of the isolation kit was added after the addition of 0.5 volumes of isopropanol.

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After pelleting the resin by centrifugation (300 xg for 5 min), the resin was washed twice with 75% RNase-free ethanol, and air dried at 50°C for 10 min. Total RNA was then eluted from the resin by resuspending the resin in 1 volume RNase-free water, vortexing vigorously for 1 min, then centrifugated at 13000 xg for 1 min. The total RNA was then transferred to a new Eppendorf tube and the resin pellet discarded.

10                    2. Poly(A)<sup>+</sup> RNA isolation:

                  Qiagen's Oligotex mRNA isolation system was used as described by the manufacturer; the procedure was repeated twice to obtain pure poly(A)<sup>+</sup> RNA. This is especially important for a random primed library to minimize the number of copies of ribosomal RNA in the cDNA. The mRNA integrity was then determined by both spectroscopy and formamide denaturing gel electrophoresis.

                  The first strand cDNA was synthesized by following the BRL cDNA synthesis protocol. To remove residual mRNA from the target cDNA, the first-strand cDNA reaction was phenol/chloroform extracted and precipitated with 2 M ammonium acetate and 3 volumes of ethanol. The cDNA/mRNA hybrids were then resuspended in 0.3 M NaOH in the presence of 2 mM EDTA and incubated at 68°C for 15 min. The hydrolysis reaction was neutralized with about 1.5 M excess of pure Tris HCl. The cDNA was then phenol/chloroform extracted and reprecipitated with 2 M ammonium acetate and 3 volumes of ethanol, rinsed with 75% ethanol, and resuspended in 7 ml of sterile water. The single strand cDNA was tailed by following the protocol of Boehringer Mannheim tailing kit.

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3. Driver mRNA preparation and photobiotinylation:

Poly(A) RNA was isolated as described above. Approximately 20 mg was then photobiotinylated twice with 20 mg photobiotin acetate (Sigma), and reconstituted at a concentration of 1 mg/ml in RNase-free water. Excess photobiotin was removed with water saturated isobutanol, and ethanol precipitated and resuspended in 30 ml DEPC-treated water.

10

4. Subtractive hybridization reaction:

The photobiotinylated driver mRNA was coprecipitated with the tester cDNA and resuspended in 2 ml RNase-free water. To allow the nucleic acids to go into solution, the preparation was left at room temperature for a few hours with intermittent gentle stirring followed by another 20 hours incubation at 68°C. Photobiotinylated driver was dissolved to a final concentration of 2mg/ml. In general, a concentration of driver RNA of at least 1mg/ml should be used.

15

20

5. Post-hybridization hybrid removal:

After the hybridization, streptavidin was added to a final concentration of 0.2 mg/ml and incubated at room temperature for 10 min. The streptavidin was then removed with a phenol/chloroform extraction. After the extraction, the cDNA was precipitated with ethanol.

25

30

A pair of primers: AGCGCTACGGTCGACCCG GCG TTT TTT TTT TTT TTT TTT (ACG)X (SEQ ID NO:15) (Sal I T21 anchored primer) and GGA AGG AAA AAA GCG GCC GCT ACA (SEQ ID NO:16) (Not I -N9 primer) were used in PCR to amplify cDNA. The expand PCR kit was used. Fifteen cycles were used to generate enough material for gel fractionation approach to allow for an equal size

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representation in the library. To allow for the annealing of the first primer, the annealing temperature of the initial five cycles of the PCR were performed at 35°C for 1 min. The cDNA representing  
5 different size fractions were fractionated on a gel. *Sal*I adapters were added to the duplex cDNA, which was then digested with *Not*I and cloned into pSport vector.

#### B. Isolation of cDNA Clone

10 The library was screened by expressed sequence tag (est) analysis. Individual clones from this library were randomly picked and sequenced on an Applied Biosystems 373A automated DNA sequencer using vector primer and Taq dye-terminator reactions (Applied  
15 Biosystems). The resulting nucleotide sequence obtained from the randomly picked clone NNT-1 was translated, then compared to the existing database of known protein sequences using a modified version of the FASTA program.

20 One clone (khjl-00008-f2) has about 21% homology at translated amino acid sequence level with CNTF. The entire insert of the cDNA clone was sequenced and found to encode a full-length clone, i.e., it contains Met at the 5' end and one stop codon  
25 upstream of Met and another stop codon at the 3' end.

The sequence of this full-length cDNA is shown in Figure 1. The predicted amino acid sequence of the protein is shown in Figure 3. The putative signal peptide spanned from amino acid -27 (Met) to  
30 amino acid -1 (Ala).

#### C. Isolation of the Genomic Clone

The genomic DNA of NNT-1 was obtained from a human genomic P1 library (Genome Systems Inc., St.  
35 Louis, MO; catalog no. P1-2535). The library was screened using the NNT-1 cDNA as a probe. The cDNA was

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radiolabeled using the Amersham Rediprime kit (Amersham, Arlington Heights, IL; catalog no. RPN-1633) and the hybridization and prehybridization solution was: 50 percent formamide, 5 X SSC, 5 X Denhardt's, 5 0.05 percent sodium pyrophosphate, 0.1 percent SDS, and 100 mg/ml salmon sperm DNA. Prehybridization was for about 1 hour, and hybridization was for about 16 hours at 42°C.

After hybridization, the filters were washed 10 in 0.2 X SSC and 0.1 percent SDS at 42°C for about 30 minutes, and then exposed to film. Two positive clones were identified, and the plasmids containing these clones were purified according to Genome Systems Inc. protocols. The plasmid DNA was then sequenced 15 directly.

The genomic sequence encoding NNT-1 is shown in Figure 2 (SEQ ID NO:3). The gene consists of 3 exons and 2 introns. The coding regions are presented in uppercase, while the noncoding regions, including 5' 20 untranslated region, introns and 3' untranslated region are presented in lower case.

Example II: Preparation of Recombinant Mammalian Protein of NNT-1

25

An expression vector containing human NNT-1 cDNA and flag-tag peptide was constructed by PCR amplification of the fusion gene. A sense primer with Hind III site at the 5' end:

30

(5' -AGCAAGCTTCACCATGGACCTCCGAGCAGGGGACTC-3')  
(SEQ ID NO: 6)

which encodes amino acid -27 (Met) to amino acid -21 35 (Asp) and an anti-sense primer with NotI site at the 5'



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end which encode for flag-tag peptide and the last 8 amino acids of the 3' end

(5' AGCGGGGCCGCACTACTTGRCATCGTCGRCGTCCTTGTACTCGAAGCCATGA  
5 GCCCCCAGGTGCAG-3') (SEQ ID NO: 7)

were used in PCR to amplify a fusion gene. The fusion gene was ligated into the P CEP4 vector (Invitrogen Inc., San Diego, CA). The expression vector was  
10 transfected into EBNA-1 293 cells with lipofectin (BRL, Gaithersburg, MD) using the manufacturer's recommended method. Forty-eight hours after transfection, both 293 cells and the conditioned medium were harvested and analyzed in Western blot by using the anti-flag-tag  
15 antibody (Eastman Kodak Co., New Haven CT). The majority of recombinant protein was found in the 293 cell lysate. Therefore, anti-flag antibody gel (Eastman Kodak Co., New Haven, CT) was used to purify the protein from the 293 cell lysate. A 28-30 kd  
20 protein was purified following the manufacturer's protocol. This recombinant protein was used in the biological function analysis (for motor neuron and sympathetic neuron survival assay). The N-terminal amino acid of the protein was determined to be Leu  
25 (amino acid 1) indicating that the potential signal peptide was cleaved (amino acid -27 to amino acid -1).

Example III: Preparation of Recombinant E coli NNT-1 Protein

30

A cDNA clone of NNT-1 encoding amino acids Leu (1) to Phe (198) of SEQ ID NO: 2 was inserted into the vector pAMG21 which is a derivative of pCFM 1656 (ATCC accession number 69576) and contains appropriate  
35 restriction sites for insertion of genes downstream from the lux PR promoter (see US Patent No. 5,169,318

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for a description of the lux expression system). The host cell used was *E. coli* K12, strain CGSC 6159 (Yale University genetic stock, New Haven, CT). The host cells were transformed with the vector using standard transformation procedures, and were then incubated in 2  
5 XYT medium containing about 50 ul/ml kanamycin at 30°C. Induction of NNT-1 gene product was commenced by adding the autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture medium to a final concentration of about  
10 30 ng/ml, and the cultures were incubated at either 30°C or 37°C for about 6 hours after which time the cells were examined by microscopy for inclusion bodies.

The majority of NNT-1 protein was found to be located in the inclusion bodies. Therefore, a cell  
15 paste was prepared by pelleting the cells. The inclusion bodies were solubilized at low pH and the protein was purified by sequential precipitation. The protein was dialyzed before loading a sample on to SDS-PAGE to assess purity. Coomassie staining of the gel  
20 indicated that the protein was at least 95 percent pure.

#### Example IV: Neurobiological function of NNT-1

##### 25 A. Chick Motor Neuron Assay

Motor neurons (MN) enriched culture from lumbar spinal cord were prepared from embryonic day E5.5 chicks. MN neurons were enriched by using a 6.8% metrizamide gradient. In brief, lumbar spinal cords  
30 were dissected, freed of meninges and DRG. Spinal cords were incubated in papain containing L15 medium (Gibco/BRL, Grand Island, NY) for 20 minutes at 37°C (Worthington Biochemical Corp, Freehold, NJ). Enzymatically softened spinal cord fragments were  
35 dissociated into single cells by pipetting. The cell suspension was then layered onto a 6.8% metrizamide

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(Serva, Feinbiochemicala, Germany) cushion, and the tube was centrifuged at 500 g for 20 minutes. The interface between metrizamide cushion and cell suspension was collected and diluted into culture medium. The fraction was then gently layered onto a 4% BSA cushion and centrifuged at 280 g for 10 minutes. The pellet was resuspended in culture medium containing L15 medium with 10% fetal bovine serum supplemented with 3.6 mg/ml glucose, 5 ng/ml sodium selenite, 6.25 ng/ml progesterone, 0.1 mg/ml conalbumin, 16 mg/ml putrescine, and 5 mg/ml insulin. 10,000 cells/well were seeded into 96 well tissue culture plates. Serial dilutions of the neurotrophic factor (NNT-1 or CNTF) were added to the culture and incubated for 3 days. At day 3, MTT was added into the culture for 4.5 hours. The formazan product was solubilized, and the plates were read at 570 wavelength with a 650 nm subtraction for visible interference. The optical density (OD) reading is proportional to the number of surviving neurons in culture. The absorbance at 570 nm (increasing neuron survival) in triplicate wells is plotted as a function of final concentration of NNT-1 or CNTF.

Results of the analysis are presented in Figure 7. The absorbance at 570nm is expressed as 1000 fold of the actual reading. The results showed that NNT-1 can support chick motor neuron growth. Its maximal activity reaches about 90% that of CNTF.

### B. Chick Sympathetic Neuron Assay

Cultures of primary chick embryo sympathetic chain ganglia were prepared. Briefly, sympathetic ganglia were removed from fertile, pathogen-free chicken eggs that had been incubated for 9 days at 37.6°C in a humidified atmosphere. The ganglia were chemically dissociated by exposure first to Hanks'

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Balanced Salt Solution without divalent cations, containing 10mM HEPES buffer pH 7.2 for 10 min at 37°C, and then by exposure to a solution of 0.125% bactotrypsin 1:250 (Difco, Detroit, Michigan) in Hanks' Balanced Salt Solution modified as above for 12 min at 37°C. Trypsinization was stopped by addition of fetal calf serum to a final concentration of 10%.

After this treatment, ganglia were transferred to a solution consisting of Dulbecco's high glucose Modified Eagle's Medium with bicarbonate contain 10% fetal calf serum and 10mM HEPES, pH 7.2 and were mechanically dissociated by trituration approximately 14 times through a 20-gauge, 1" double-hubbed stainless steel needle.

The dissociated ganglia were then plated in culture medium (Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 4mM glutamine, 60mg/L penicillin-G, 25mM HEPES, pH 7.2) in 100 mm diameter tissue culture dishes (approximately 40 dissociated ganglia per dish) for two to three hours. This preplating was done in order to separate the nonneuronal cells, which adhere to the dish, from the nerve cells, which do not adhere. After preplating, the nonadherent nerve cells were collected by centrifugation, resuspended in culture medium, and plated in 50 ml per well onto half area 96-well microtiter tissue culture plates at a density of 2500 nerve cells per well. The microtiter wells had been previously exposed to a 1 mg/ml solution of poly-L-ornithine in 10mM sodium borate, pH 8.4 overnight at 4°C, washed in sterile purified water and air-dried.

Final concentrations of neurotrophic factors to which the cells were exposed are as follows: 1) for the CNTF standard, nine-point serial dilution curves ranged from 100 ng/ml to 6 pg/ml; 2) for the NNT-1 protein, nine-point serial dilutions curves ranged from

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100 ng/ml to 0.12 pg/ml. Twenty-five ml of a serial dilution of the sample to be assayed for neurotrophic activity was added to each well and the dishes were incubated for 38-46 hours at 37°C in a humidified atmosphere containing 7.5% CO<sub>2</sub>. Then 18 ml per well of a 1.5 mg/ml solution of the tetrazolium dye MTT in Dulbecco's high glucose Modified Eagle Medium with bicarbonate contain 10mM HEPES, pH 7.2 was added, and the cultures were placed in the 37°C incubator for 4.5 hours. Then 75 ml of a solution of 50% N,N-dimethyl formamide containing 20% sodium dodecyl sulfate, pH 4.7 was added to dissolve the crystalline formazan product and the plates were incubated in the 37°C incubator for a minimum of 12 hours. The absorbance at 579nm was determined relative to a 650nm reference for each well using an automatic microtiter plate reader. The resulting absorbance is proportional to the number of living cells in each well, defined as those nerve cells capable of reducing the dye.

Results of the analysis are presented in Figure 8. The results demonstrate that NNT-1 supports chick sympathetic neuron growth.

#### Example V: Northern Blot Analysis of Tissue Distribution

Northern blots of human tissues were purchased from Clontech (Palo Alto, CA). The Northern blots were probed with a human NNT-1 cDNA probe. Two cDNA fragments spanning the 5' and 3' coding region of NNT-1 were labeled and used as a probe to analyze the tissue expression of the NNT-1 gene. The result showed that NNT-1 was expressed as a 2.2 kb transcript in the tissues of spleen, lymph node and peripheral blood lymphocytes, bone marrow and fetal liver, kidney, lung, colorectal adenocarcinoma cells SW480, Hela cell S3, lung carcinoma A 549, chronic myelogenous leukemia K-562

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cells, Burkitt's lymphoma Raji cells. The tissue distribution of the gene suggests that the gene may be also involved in development of the immune system or of hematopoietic cells.

5

Example VI: Chromosome localization of the NNT-1 gene

Chromosome localization of the gene was performed by FISH. A 14 kb genomic fragment was  
10 biotinylated with dATP using BRL BioNick labeling kit. (15 C 1 hour). The procedure for FISH was performed according to Heng *et al.*, *Proc Nat Acad Sci USA* 89:9509-9513, 1992. The result showed that the gene is located on chromosome 11 q13 which is close to the  
15 human CNTF gene locus (chromosome 11 q12).

Example VII: Isolation of mouse cDNA clone

A mouse partial cDNA clone was isolated by  
20 PCR amplification from the mouse 11 day-embryo cDNA (Clontech, Palo Alto, CA) using the human specific primer. The full-length cDNA clone was further obtained by 5' RACE and 3' RACE. The mouse cDNA nucleotide sequence and amino acid sequence are shown  
25 in Figs. 4 and 5, respectively. The mouse protein shares 96% identity with the human protein, indicating that the protein is highly conserved throughout evolution. Like the human protein, the mouse protein also contains a potential N-linked glycosylation site  
30 at amino acid 2 (Asn).

Example VIII: Comparison of NNT-1 with other members of the family

35 The amino acid sequence of NNT-1 suggests that the protein belongs to the family of CNTF (SEQ ID NO:12),

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which includes IL-11 (SEQ ID NO:8), IL-6 (SEQ ID NO:9),  
cardiotrophin (SEQ ID NO:11), oncostatin (SEQ ID NO:13)  
and granulocyte colony-stimulating factor (G-CSF) (SEQ ID  
NO:10). We compared the amino acid sequence of NNT-1 with  
5 all of the members of the family by the computer program  
PILEUP and the results are shown in Fig. 6. As with all  
the other members of this family, the secondary structure  
of the NNT-1 protein was predicted to contain four anti-  
parallel alpha-helices.

10

Example IX: Phenotype of NNT-1 Transgenic Mice

A. Phenotype of NNT-1 Transgenic Mice

The protein encoded by the NNT-1 gene has  
15 some homology to CNTF and *in vitro* activity in bone  
marrow and nerve cell assays. Studies of mice  
transplanted with NNT-1 transfected bone marrow  
demonstrated mild lymphoproliferation in  
gastrointestine-associated lymphoid tissues, but no  
20 other obvious phenotypic changes.

Materials and Methods

Species: Mouse      Strain: BDF1      Age: 17 wks (120 days)  
Test article: NNT-1 (WX240)      Sex: Male/Female

25

Treatment Groups

GROUP	MOUSE NO.
Negative	22, 23, 45, 63, 65
Positive	35, 36, 46, 60, 62

There were no obvious abnormalities detected  
30 in the two groups.

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Gross necropsy was performed with selected tissues fixed in buffered zinc formalin for histopathologic examination [brain, heart, kidneys, adrenals, duodenum, pancreas, bladder, liver, lungs, spleen, any gross lesions]. Tissues were fixed overnight before routine histologic processing. The data were analyzed using the *JMP* (SAS Institute, Cary, NC) software program.

Tests: organ weights, body weight, histopathology, immunohistology, Northern blot.

The following treatment-related changes were present in the NNT-1-transgenic mice:

The spleen had moderate to marked reactive lymphoid hyperplasia (FIG. 10) involving the follicular (B cell) and periarteriolar (T cell) areas in the transgenic mice. The lymphoid hyperplasia was most prominent in the high expresser mouse #62 (FIG. 10), and correlated well with the splenomegaly seen at necropsy. The other high expresser mouse #60 had only mild hyperplasia of the lymphoid areas accompanied by massive diffuse extramedullary hematopoiesis of all three lineages. Although it is difficult to make any general conclusions about the splenic effects of NNT-1 on the basis of these two high expresser mice, the lymphoproliferation seen in mouse #62 is in agreement with our findings with the injected protein (See Example X A below), while the EMH found in mouse #60 may reflect an *in vivo* correlate of the previous *in vitro* bone marrow culture findings.

The liver of mouse #60 had multifocal aggregates of lymphocytes and plasma cells infiltrating perivascular spaces and expanding into the adjacent sinusoids in a peculiar pattern that resembled intrahepatic "islands of lymphopoiesis" (FIG. 12). By immunohistochemistry, the lymphoid aggregates were

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composed of B220+ cells and CD3+ cells. Similar but milder and typically perivascular lymphoid infiltrates were also found in mouse #62. Other changes found in the liver occurred sporadically in individual mice in the control and/or transgenic groups.

The gastrointestinal tract had minimal to moderate reactive lymphoid hyperplasia of Peyer's patches (gut-associated lymphoid tissue). Similarly, the cervical and mesenteric lymph nodes were more reactive in the transgenic mice than in the controls, although this change was not as prominent a feature of this study than our study with injected NNT-1 protein (See Example X A below).

The bone marrow, central and peripheral nervous systems of the transgenic mice appeared normal. Generally, the changes in the other tissues were sporadically found in one or more animals in the negative control and/or transgenic groups, and were not interpreted to be transgene-related.

The data from this study indicate that the NNT-1 transgenic mice have an interesting phenotype characterized by proliferation of T and B lymphocytes and plasma cells in multiple peripheral tissues, including the spleen, lymph nodes, gut-associated lymphoid tissue, kidneys and liver. NNT-1 may also induce extramedullary hematopoiesis in some peripheral tissues, such as the spleen and pancreas, in the absence of significant changes in the peripheral blood or bone marrow. Thus, the data from the NNT-1 transgenic mice generally support the findings from our 7-day mouse study with injectable NNT-1 protein (Example X A below), which induced proliferation of lymphoid tissues without detectable effects on bone marrow or central nervous system.

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Interestingly, the glomerulonephritis detected in the two high expresser NNT-1 transgenic female mice closely resembles the spontaneous glomerulonephritis seen in the MRL/lpr (Fas-deficient) mice, which develop an early-onset SLE-like autoimmune syndrome associated with polyclonal B-cell activation, multiple autoantibodies, circulating immune complexes and accumulation of an unusual population of double negative (CD4- CD8- TCRab+ CD3+) T cells that also express the CD45R isoform called B220+, which is normally a marker of B cells (Singer et al., *Curr. Opin. Immunol.*, 6:913-920, 1994). Moreover, some of the biologic effects of NNT-1 also mimic those of interleukin-6, which (like CNTF, LIF and IL-11) utilizes the gp130 signaling transducer and has pleiotropic effects on the liver, kidney, brain, skin, immune and hematopoietic systems (Ryffel et al., *Int. Rev. Exp. Pathol.*, 34A:79-89, 1993). Therefore, it will be important to determine if the lymphocytes found in the peripheral blood or tissues might have an unusual phenotype with dual expression of T and B cell markers by flow cytometric analysis.

B. FACS Immunophenotyping of NNT-1 Transgenic  
Founders

Tissues analyzed

Peripheral blood samples were obtained via retro-orbital bleeds. Nine samples from each group of founder littermate control and NNT-1 positive (by PCR) mice were received; none were clotted. Approximately 20-40ul of blood per sample was incubated first with Fc block antibody followed by fluorescent antibodies for various cell surface antigens.

Antibodies were chosen for markers to differentiate most hematopoietic cell populations in

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circulating peripheral blood. Also, some B and T-cell activation/differentiation markers were chosen based on origin of library for this expressed sequence tag (est). The library was created from Jurkat cells (a T-cell line) activated with toxic shock syndrome toxin (TSST).

### Antibodies

- Fc Block (CD32/16) - as part of pre-incubation to block non-specific binding, a total of 21 antibodies were used. Data was analyzed as single color histograms.
- Rat IgG fluorescein isothiocyanate (FITC) + Rat IgG phycoerythrin (PE)
- Ham IgG FITC + Ham IgG PE
- CD45 FITC + GR-1 (CD97) PE ----- Pan leukocyte vs granulocyte
- CD4 FITC + CD8 PE ----- T-cell subsets Helper vs killer
- Th1.2 FITC + B220 PE ----- T-cell vs pan B-cell marker
- CD69 FITC + CD28 PE ----- Activation markers for T & B or just T-cells
- CD3 FITC + CTLA4 PE ----- Pan T-cell vs T-cell activation
- ckit FITC + Sca-1 PE ----- myeloid and progenitor cells vs progenitors and peripheral lymphocytes
- CD40 FITC + CD40L PE ----- B-cell diff. Ag vs T-cell ligand for same
- CD62L FITC + CD54 PE ----- Activating adhesion molecules on B and T-cells
- CD34 FITC (data not analyzed)

### Results

- A pronounced increase was observed in absolute cell numbers for four of the NNT-1 positive animals for B220+, CD40+, CD62L+, and CD54+ cells.

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These four animals (#24,35,60,62) were later confirmed as expressers by Northern blot. The increase in B220+ and CD40+ cells ranged from 2-4 fold above the control. CD62L+(LECAM) and CD54+(ICAM) ranged from 1.5-3 fold above the control group. Markers showing an increase in three of the four expressers included Sca-1 (2-6 times control) and ckit (2-3 times control). Additional markers including CD3, CD4, CD8, Thy1.2 showed modest increases in two of the four expressers, though not in a consistent fashion (although these are all T-cell markers, they were not all positive in the same expressers). GR1 showed an increase in one of the expressers, but there was an even higher GR1+ cell number in one of the control animals, so this is probably not significant. The rest of the antibodies were either not positive, not significantly different, or in the case of CD34, impossible to analyze.

#### Summary

A very definite increase in the absolute number of circulating lymphoid cells is observed in these mice. This increase in the lymphoid population seems to consist primarily of B-cells, although some increase in T-cell numbers may be seen as well. Neither lymphoid population appears to exhibit an increase in activated cell types. Little to no effect is seen on the circulating myeloid cell population. Increases in ckit and Sca-1 do not necessarily correlate to an increase in progenitor cells as these markers are found on mature circulating cells as well.

The data is suggestive of a B-cell directed proliferation as these cell numbers all correlate well with expression. The increases in some of the animals' T-cells could possibly be a secondary effect of some other factor(s) being produced by the increased B-cells. One interesting observation with regard to the

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B-cells is a slight but very consistent difference between the number of B220+ cells and CD40+ cells. Although both of these are B-cell markers, CD40 is also found on dendritic cells as well.

5

Example X: Lymphoid Hyperplasia in Mice Injected with NNT-1

- 10 A. A Seven-Day Exploratory Intravenous/Subcutaneous Study in NNT-1 Treated BDF1 Female Mice
- The protein encoded by NNT-1 had some homology to CNTF and *in vitro* activity in bone marrow and nerve cell assays. The objective of this study was to determine the systemic effects and potential
- 15 toxicity of NNT-1 protein when administered daily to mice for 7 days.

Materials and Methods

20 Twenty 6-week old, female BDF1 mice were used for the study. The mice were randomly assigned into the following treatment groups (n=5/group):

1. PBS buffer control (intravenous dosing once daily for 7 days)
2. NNT-1 at 1.5 mg/kg (intravenous)
- 25 3. NNT-1 at 0.15 mg/kg (intravenous)
4. NNT-1 at 1.5 mg/kg (subcutaneous)

The mice were not fasted prior to gross necropsy. One hour prior to necropsy (24 hrs after last dosing), the mice were given an intraperitoneal

30 injection of BrdU (at 50 mg/kg for cell proliferation studies). Blood was obtained via cardiac puncture for the determination of hematology (hemoglobin, hematocrit, red blood cell count, platelet count, mean platelet volume, total and differential leukocyte

35 counts) and clinical chemistry parameters (alanine aminotransferase, aspartate aminotransferase, alkaline

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phosphatase, lactate dehydrogenase, glucose, urea nitrogen, creatinine, total protein, albumin, globulin, calcium, phosphorus, total bilirubin, uric acid, cholesterol and triglycerides).

5           Gross necropsy was performed with selected tissues fixed in buffered zinc formalin for histopathologic examination [adrenals, bone marrow, bone (femur), brain, cecum, proximal and distal colon, duodenum, esophagus, heart, ileum, jejunum, kidneys, 10 liver, lungs, mammary glands, ovaries, pancreas, skeletal muscle, skin, spleen, stomach, thymus, thyroid glands, trachea, urinary bladder, uterus, vagina, white and brown adipose tissue, any gross lesions]. Tissues were fixed overnight before routine histologic 15 processing. Organ weights were obtained for the spleen, liver, stomach, kidneys and thymus.

### Results

Spleen. There was prominent lymphoid 20 hyperplasia in the white pulp of the spleen with enlargement of the periarteriolar lymphoid sheaths (T-cell areas) and follicles (B-cell areas) in the NNT-1-treated groups. However, the extent of extramedullary hematopoiesis was not apparently increased in these 25 groups, which suggests that this protein may have stimulatory or growth factor-like effects on lymphocytes rather than on hematopoietic cells in vivo.

Lymph node. The NNT-1-treated mice had mild to marked reactive lymphoid hyperplasia of the 30 follicular (B-cell) and paracortical (T-cell) areas of the lymph node cortex. Although this change may reflect an early immune response to the recombinant protein, the degree of generalized reactive lymphoid hyperplasia that was present in the spleen, lymph 35 nodes, Peyer's patches and bone marrow suggests that

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this may be a specific treatment-related effect of NNT-1:

#### Summary and Conclusions

5           The most significant finding derived from this study was that NNT-1-treatment of mice for 7 days appeared to induce proliferation of lymphoid tissues, particularly in the spleen and lymph nodes. However, this protein did not appear to have any detectable  
10 effects on the hematopoietic or central nervous systems under the conditions of this study.

#### B. FACS Analysis of NNT-1 Injected Mice

Reagents and Mice. Recombinant human NNT-1  
15 and rhIL-1 were from Amgen Inc., Thousand Oaks, CA. LPS (*Escherichia coli* 0111:B4) was purchased from LIST Biologic Laboratories, Campbell, CA. Female Balb/c mice of approximately 20 g were purchased from Charles River Laboratories, Wilmington, MA. Mice were housed  
20 in rooms maintained at constant temperature and humidity and subjected to 12 hour light/dark cycle. Mice received standard laboratory diet and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with institutional  
25 guidelines that are in compliance with national and international laws and policies (U.S. National Research Council, Guide for the Care and Use of Laboratory Animals, 1996).

Lymph Node Weight and Cell Counts. For seven  
30 consecutive days mice received a daily i.p. injection of 5 mg/Kg of NNT-1 or buffer. Twenty-four hours after the seventh injection, mice were sacrificed for the collection of peripheral (cervical and axillary) lymph nodes. Lymph nodes were pooled, weighed and  
35 homogenized so as to prepare a cell suspension. Cells were then counted with a Sismex cell counter (Toa

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Medical Corporation, Kobe, Japan), stained by direct IF using a rat anti-mouse anti-CD45R (anti-B220) MAb (Pharmingen, San Diego, CA) and analyzed in a FACSCAN using the Cell Quest software (Becton and Dickinson, San Jose, CA).

Statistical Analysis. Results are expressed as mean $\pm$ SD. TNF values were log-transformed to lessen their skewed distribution and bring them to normality. The Shapiro-Wilks test was used to analyze the normality of their distribution before and after transformation. Differences between groups were analyzed by the Student's t test. Since BW was repeatedly measured in each individual, differences in BW within and between groups were tested by the analysis of variance (ANOVA) for repeated measures.

Lymph Node Weight and Cell Counts. NNT-1 treatment increased the counts of total and CD45-positive cells in peripheral lymph nodes (FIG. 17).

#### 20 Example XI: NNT-1 Shows In-Vivo Activities Characteristic of Cytokines of the IL-6 Family

Reagents, mice and statistical analyses are as set forth in Example X B above.

25 Serum amyloid A (SAA) Induction, Potentiation of Corticosterone and IL-6 Induction by IL-1 and Inhibition of LPS-Induced TNF. NNT-1 was given i.p. at a dose of 5 mg/kg, alone or in association with IL-1 (100 ng/mouse) or LPS (100 ng/mouse). Control mice received the solvent for NNT-1 (10 mM acetate in saline). Blood was taken from the retro-orbital plexus 8 hours after the administration of NNT-1 or saline for SAA determination, 2 hours after for corticosterone and IL-6 and 1.5 hours after for TNF. Experiments were conducted on groups of five or ten mice.



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SAA, IL-6 and TNF were measured in serum by ELISA using commercially available kits (Biogen, Camarillo, CA); results were expressed in  $\mu$ g, ng and pg/ml, respectively. Corticosterone was measured by  
5 RIA using a commercially available kit (ICB Biomedical, Costa Mesa, CA); results were expressed in ng/ml.

SAA Induction, Potentiation of Corticosterone and IL-6 Induction by IL-1 and Inhibition of LPS-  
10 Induced TNF. NNT-1 induced circulating SAA (FIG. 13).

NNT-1 potentiated the induction by a low dose of IL-1 of either serum corticosterone or IL-6 (FIGS. 14 and 15). NNT-1 also showed the ability to increase the circulating levels of corticosterone when it was  
15 injected alone.

NNT-1 inhibited the induction by LPS of serum TNF (FIG. 16).

#### Summary of Results

20 Inflammatory processes are accompanied by the production of TNF, a cytokine largely responsible for the tissue damage and functional impairment that distinguish inflammation-related pathology. Often IL-1 is co-produced with TNF and is also thought to be a  
25 pathogenetic mediator during inflammation.

Corticosteroids are broad spectrum and very powerful anti-inflammatory agents which are induced by IL-1 via an efficient negative feed-back circuit.

Corticosteroids inhibit both TNF and IL-1 production.  
30 IL-6, which is also induced by both TNF and IL-1, is also able to inhibit TNF and IL-1 production via another negative feed-back circuit.

The ability of NNT-1 to induce corticosteroids and IL-6, at least in presence of IL-1,

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suggests that this molecule has the ability of potentiating two physiological anti-inflammatory circuits. This may lead to an accelerated inhibition of the production of TNF and IL-1 and to an accelerated resolution therefore of inflammatory processes. In addition to and independently of the induction of corticosteroids and IL-6 production, NNT-1 exhibits the property of directly blocking TNF production. This interestingly adds to the anti-inflammatory features outlined above.

#### Deposit of DNA

*E. coli* cells DH10B containing the vector P1 encoding human genomic DNA for NNT-1 (NNT-g-PI) and *E. coli* cells DH10B containing the vector PSPORT encoding human cDNA for NNT-1 have been deposited with the ATCC (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA) on January 21, 1997 and assigned accession numbers 98294 and 98295, respectively.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: CHANG, MING-SHI  
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SARMIENTO, ULLA  
SENALDI, GIORGIO
- (ii) TITLE OF INVENTION: THE NEUROTROPHIC FACTOR NNT-1
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: AMGEN INC.
  - (B) STREET: ONE AMGEN CENTER
  - (C) CITY: THOUSAND OAKS
  - (D) STATE: CA
  - (E) COUNTRY: USA
  - (F) ZIP: 91320
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/792,019
  - (B) FILING DATE: 03-FEB-1997
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: COOK, ROBERT R.
  - (B) REGISTRATION NUMBER: 31,602
  - (C) REFERENCE/DOCKET NUMBER: A-442B

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 797 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 90..764
- (ix) FEATURE:
  - (A) NAME/KEY: mat\_peptide
  - (B) LOCATION: 171..764
- (ix) FEATURE:
  - (A) NAME/KEY: sig\_peptide

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(B) LOCATION: 90..170

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATTAAAGCTT CGCCGGAGCC GCGGCTCGCC CTCCCACTCC GCCAGCCTCC GGGAGAGGAG	60
CCGCACCCGG CCGGCCAGC CCCAGCCCC ATG GAC CTC CGA GCA GGG GAC TCG	113
Met Asp Leu Arg Ala Gly Asp Ser	-20
-27 -25	
TGG GGG ATG TTA GCG TGC CTG TGC ACG GTG CTC TGG CAC CTC CCT GCA	161
Trp Gly Met Leu Ala Cys Leu Cys Thr Val Leu Trp His Leu Pro Ala	-5
-15 -10	
GTG CCA GCT CTC AAT CGC ACA GGG GAC CCA GGG CCT GGC CCC TCC ATC	209
Val Pro Ala Leu Asn Arg Thr Gly Asp Pro Gly Pro Gly Pro Ser Ile	10
1 5	
CAG AAA ACC TAT GAC CTC ACC CGC TAC CTG GAG CAC CAA CTC CGC AGC	257
Gln Lys Thr Tyr Asp Leu Thr Arg Tyr Leu Glu His Gln Leu Arg Ser	25
15 20	
TTG GCT GGG ACC TAT CTG AAC TAC CTG GGC CCC CCT TTC AAC GAG CCA	305
Leu Ala Gly Thr Tyr Leu Asn Tyr Leu Gly Pro Phe Asn Glu Pro	45
30 35 40	
GAC TTC AAC CCT CCC CGC CTG GGG GCA GAG ACT CTG CCC AGG GCC ACT	353
Asp Phe Asn Pro Pro Arg Leu Gly Ala Glu Thr Leu Pro Arg Ala Thr	60
50 55	
GTT GAC TTG GAG GTG TGG CGA AGC CTC AAT GAC AAA CTG CGG CTG ACC	401
Val Asp Leu Glu Val Trp Arg Ser Leu Asn Asp Lys Leu Arg Leu Thr	75
65 70	
CAG AAC TAC GAG GCC TAC AGC CAC CTT CTG TGT TAC TTG CGT GGC CTC	449
Gln Asn Tyr Glu Ala Tyr Ser His Leu Leu Cys Tyr Leu Arg Gly Leu	90
80 85	
AAC CGT CAG GCT GCC ACT GCT GAG CTG CGC CGC AGC CTG GCC CAC TTC	497
Asn Arg Gln Ala Ala Thr Ala Glu Leu Arg Arg Ser Leu Ala His Phe	105
95 100	
TGC ACC AGC CTC CAG GGC CTG CTG GGC AGC ATT GCG GGC GTC ATG GCA	545
Cys Thr Ser Leu Gln Gly Leu Leu Gly Ser Ile Ala Gly Val Met Ala	125
110 115 120	
GCT CTG GGC TAC CCA CTG CCC CAG CCG CTG CCT GGG ACT GAA CCC ACT	593
Ala Leu Gly Tyr Pro Leu Pro Gln Pro Leu Pro Gly Thr Glu Pro Thr	140
130 135	
TGG ACT CCT GGC CCT GCC CAC AGT GAC TTC CTC CAG AAG ATG GAC GAC	641
Trp Thr Pro Gly Pro Ala His Ser Asp Phe Leu Gln Lys Met Asp Asp	155
145 150	
TTC TGG CTG CTG AAG GAG CTG CAG ACC TGG CTG TGG CGC TCG GCC AAG	689
Phe Trp Leu Leu Lys Glu Leu Gln Thr Trp Leu Trp Arg Ser Ala Lys	170
160 165 170	
GAC TTC AAC CGG CTC AAG AAG AAG ATG CAG CCT CCA GCA GCT GCA GTC	737
Asp Phe Asn Arg Leu Lys Lys Lys Met Gln Pro Pro Ala Ala Ala Val	185
175 180 185	
ACC CTG CAC CTG GGG GCT CAT GGC TTC TGACTTCTGA CCTTCTCCTC	784

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Thr Leu His Leu Gly Ala His Gly Phe  
190 195

TTCGCTCCCC CCC

797

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 225 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp Leu Arg Ala Gly Asp Ser Trp Gly Met Leu Ala Cys Leu Cys  
-27 -25 -20 -15

Thr Val Leu Trp His Leu Pro Ala Val Pro Ala Leu Asn Arg Thr Gly  
-10 -5 1 5

Asp Pro Gly Pro Gly Pro Ser Ile Gln Lys Thr Tyr Asp Leu Thr Arg  
10 15 20

Tyr Leu Glu His Gln Leu Arg Ser Leu Ala Gly Thr Tyr Leu Asn Tyr  
25 30 35

Leu Gly Pro Pro Phe Asn Glu Pro Asp Phe Asn Pro Pro Arg Leu Gly  
40 45 50

Ala Glu Thr Leu Pro Arg Ala Thr Val Asp Leu Glu Val Trp Arg Ser  
55 60 65

Leu Asn Asp Lys Leu Arg Leu Thr Gln Asn Tyr Glu Ala Tyr Ser His  
70 75 80 85

Leu Leu Cys Tyr Leu Arg Gly Leu Asn Arg Gln Ala Ala Thr Ala Glu  
90 95 100

Leu Arg Arg Ser Leu Ala His Phe Cys Thr Ser Leu Gln Gly Leu Leu  
105 110 115

Gly Ser Ile Ala Gly Val Met Ala Ala Leu Gly Tyr Pro Leu Pro Gln  
120 125 130

Pro Leu Pro Gly Thr Glu Pro Thr Trp Thr Pro Gly Pro Ala His Ser  
135 140 145

Asp Phe Leu Gln Lys Met Asp Asp Phe Trp Leu Leu Lys Glu Leu Gln  
150 155 160 165

Thr Trp Leu Trp Arg Ser Ala Lys Asp Phe Asn Arg Leu Lys Lys Lys  
170 175 180

Met Gln Pro Pro Ala Ala Ala Val Thr Leu His Leu Gly Ala His Gly  
185 190 195

Phe

## (2) INFORMATION FOR SEQ ID NO:3:

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## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5087 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 137..138
- (D) OTHER INFORMATION: /product= "INTERVENING UNSEQUENCED REGION OF >1KB"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AACCTGCGAG TGGGCCTGGC GGATGGGATT ATTAAGCTT CGCCGGAGCC GCGGCTCGCC	60
CTCCCACTCC GCCAGCCTCC GGGAGAGGAG CCGCACCCGG CCGGCCCAGC CCCAGCCCCA	120
TGGACCTCCG AGCAGGTGA AAACCCAAAC TAGCCCTGCT CTTCATAACA TGACAAGCAG	180
CGCCCCATCT GATACCTAAA CCGACCAAGT CACAGCCCTC CAACTCACCC TCTGCCTGCC	240
CAGACCTCAC CACATCCTTG TGGACTCAA CCTCAACCGC ACTAAATCAA CCAAATCCCA	300
AGTCTAAACT AATCTGAAAC TTTTAAAGTA ACCCAGTCCT TAAACCTAAC CTAGCCCAAT	360
GCCAATTATA TCTACCCTAG CCAAACCCTA ACTGCCTTTG CCAGTCCAAA GTGTCCACTG	420
AATCCTCACC TTGGTCCTCA CTGAAAATCC CAGAAAAGCA TATTTCCCCA CTGCCCACAT	480
CCCTCCTTAC AGCACCCAAC CCTGGCCTCT GGAATCCTGG TATCCTGGGA TGTCCAAACT	540
CTGCAGTGCC ATCAGCCAAC AAGCCCGACT CGTCAAATGC ACCTCTCTCC CTTCTGTCC	600
CCACCCTTGC AGGCTGATGG AAAGGCCTCA TTGAAGTCCA ACTTTTCCCC ACCTAACACC	660
AAGAACGGGG TGAACCTCCA CACTGCCACC GTTCCCTGAG AGTGAGCACT AAATCTCCTT	720
CAATCTAACC CCACCCTACA CTTCCACAC TCAGGAATCA CATCCTAGAA TATACCCAAA	780
ACTAAGCCCC ATAAGGCAGC CCGACCCTAG TGGTCTAACC CTATACCTTG CTTCTATGG	840
GTGAGTCTGT TCTTGGCGGC CGCCTCTCTC CTGCTTCCTC CCTTAGAGCT GACTGTGCTC	900
AGCCTGCCAG CTCTGACATG TGCTGTCTCC CACCCTCTGA CTCCCCTCAA GCTGCAGTGG	960
GACTGGAAGA CTGGCAGGAA GCTAGGGTAC AACTGGAACA CAGGCAGGTC GACCTGCAGT	1020
CCCTAGGCCT GGCCCCGTCC CTCCATGTAC ACACATATAC ATGTTGGCAC ACACACAGTG	1080
GCACACATGC CAAAGACTCT CTCAGCTGAC ACACAGATCC ATTCTCAAGT ATCTACTGAT	1140
AGACACTCAT GCGTGCCAAG TCCTCATCCT CAAACATACA CATGCCTCTC TTTCTCTCCC	1200
GTCTTGCCAG GAGTGTTCCT CCTCCTCCAT CCCCTCTGCC TCCCATCTGG TGTCCACCC	1260
TCACCCCCCA CCCAGCCCCA GGTGGGGACA GACACCTGAG GGGCTGCCAG CTGCTTCCCC	1320
GTGTGGGCCC GGGCCGCGCT CATGCTTCTC GTCCATCCTG CCCACAGGGG ACTCGTGGGG	1380

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GATGTTAGCG TGCCTGTGCA CGGTGCTCTG GCACCTCCCT GCAGTGCCAG CTCTCAATCG	1440
CACAGGGGAC CCAGGGCCTG GCCCCCTCCAT CCAGAAAACC TATGACCTCA CCCGCTACCT	1500
GGAGCACCAA CTCCGCAGCT TGGCTGGGAC CTATGTGAGT ATCCAGCGTA GGAATCTGGG	1560
AGTTGGGGAG GAGTGAGGAG TTGGGGAAAAG ACAGTCCTAA CCGTGGAGGG TTCTGGTAAA	1620
TGATGGGGTG AGGAGGGGCT CTTTGGCTCC CACCAGTCCC CCTGTCTGGT CTATCTCCTG	1680
CCCTTCCCTC TTAGGTGGCC CCCCCACTTC CCCATCCCTG GCCCCAGGAC TAGGCATGTG	1740
GGCAGGCCTC GCACCCGCCT TGGCCCATTG CCCCCTGGC TGCCAGCCCA GCCGCCGCC	1800
TCCCCCTGGG GGCCGGGGAA GTCTCCTCTG TTTACACCGT GTTGTGGTGT CTCTGCGCG	1860
GGCGGGGTTG GGTGGGGACA GAGGGGCCCC ACCTCCCATG CCTGCGTTCC AGCTCGCCTC	1920
TGCCCCCAGA CCTGGGGCCC TGCTGCTCTG GACCCAGGGG CCTCCCTTCC GTCTGCCTCT	1980
CCCATCCTAG CTGGGCCTCC TAGGGGGGTC ATGGGGGAAG GGGACTGTAG GGAACCCAGG	2040
CAGTAGTGGC AGGGGGTTTA GGGTGTGGAT GGAGGTTATG CTGTAAGGAT TTGGGGGTGG	2100
TCCAGAGGTG TTCAGAGAGC CCAGGAGAGA AGGAAGGAGG GTTGGAGGAG CCGAGGCACC	2160
ATGGGGAACC GGCCCCCTCT TCCCGTGTTC CTCTTCCACA TCCCAGACCC TACTCTGGAG	2220
CCAGGGAAAG AAAAGGGAAG AAGGTGGCGG GGGAGCTGGC TCCAGCCCCA GGATACACCG	2280
AGGAAATTAG TTTGTCTCTG TGCTTGTGAG CGTGTGAACC TCCCCCTGGG CCCTTGCTTA	2340
TCCCAGGCCT CTCCCCCTGC TTCTCCCTTC TTTCCCAGTT ATACATCTCC CTCATCCCTT	2400
TCCCTGGGCC CCAGCCGCTC CCCCCAGGGT TGGAAAGGGC TCTGCCCTCT TCCCTATACC	2460
ATGCTGTCTT CCATAGCCTT CCTCCTGTCC TACTCATGAG ACTGCCTCCA TTTCTTCCTT	2520
CTGCAACCCT GCTCCTATCA GCTGAACCCT TCTTTCGGAG TGTTAGTGAG TACCCGTCTC	2580
TCCCCAGCCC CTCAGCTGGT GGGCCTGGGT GTGTCAGCGG CAAATGGGGC TCTGGTTCCA	2640
ATGGGCCACT CTCATCTCTC TCTTGTTCCT TGTGCAGAAA ACCTTTGCTT CACTCCACTG	2700
CCCTCTCTAG TTCCCGACCC TTTTCTCTC CTGGCTTTCC CTGCCAAATT TCTCCAAGGA	2760
GTGGTCTACA CCCTCTGCCT CCACTTCCTC TCCAACCACT CACTTCTTAA CCCCCTGCAA	2820
TCTGGCTTCC AGGCCCCAGC AATGGTTCTC TCCAAGGTCG TCAGGCACCT CTTGCCAAG	2880
CCCGACAGTG TTTGAAGGC TCATTCTCCT TGCTGTCTGT TTTGCAGCCA CACTGCTGAG	2940
CGCTGCTGCC TTCTCGAACT CCTCTTCCTT GGTCTCTGCA CTCTCCTGGG CCACCTTCTA	3000
CCTCTCCAGC TCCTCCAGGC TCCTCTTCCT CTCTGTCTCTG CCCCCACAGC GGGCACTCTC	3060
CCAAGGTTTG CCCACCCAGC CAATCAGCAC GTCCTTCCTG AGCGTCTTGT GCGTCTCCTC	3120
CTCCTCCTTT TTCTACGCCT CTCCATTGGA GAGCTACCA CCGCCACTGC TTCAACTGTC	3180
ACCTGCATAC AAATGATATC CTTATTGGAA AACTCAGGG AGGCCATGAA CAAAGAAGCC	3240
TAGCATGGAG ACAGGGCCAG TGTCAGGGGA CACAAAAAAT AGAAACTTTG GGAGCAGGTA	3300

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TCTCCTTGGT GGTGAGCCAG CGGCTCTGCC CTCCTCCTTC CCCATCACCC TCTCCTTTTC	3360
ACAGCTGAAC TACCTGGGCC CCCCTTTCAA CGAGCCAGAC TTCAACCCTC CCCGCCCTGGG	3420
GGCAGAGACT CTGCCCAGGG CCACTGTTGA CTTGGAGGTG TGGCGAAGCC TCAATGACAA	3480
ACTGCGGCTG ACCCAGAACT ACGAGGCCTA CAGCCACCTT CTGTGTTACT TGCCTGGCCT	3540
CAACCGTCAG GCTGCCACTG CTGAGCTGCG CCGCAGCCTG GCCCACTTCT GCACCAGCCT	3600
CCAGGGCCTG CTGGGCAGCA TTGCGGGCGT CATGGCAGCT CTGGGCTACC CACTGCCCCA	3660
GCCGCTGCCT GGGACTGAAC CCACTTGGAC TCCTGGCCCT GCCCACAGTG ACTTCCTCCA	3720
GAAGATGGAC GACTTCTGGC TGCTGAAGGA GCTGCAGACC TGGCTGTGGC GCTCGGCCAA	3780
GGACTTCAAC CGGCTCAAGA AGAAGATGCA GCCTCCAGCA GCTGCAGTCA CCCTGCACCT	3840
GGGGGCTCAT GGCTTCTGAC TTCTGACCTT CTCCTCTTCG CTCCTCCTTC AAACCCTGCT	3900
CCCACTTTGT GAGAGCCAGC CCTGTATGCC AACACCTGTT GAGCCAGGAG ACAGAAGCTG	3960
TGAGCCTCTG GCCCTTTCCT GGACCGGCTG GCGTGTGAT GCGATCAGCC CTGTCTCCTC	4020
CCCACTTCCC AAAGGTCTAC CGAGCTGGGG AGGAGGTACA GTAGGCCCTG TCCTGTCCTG	4080
TTTCTACAGG AAGTCATGCT CGAGGGAGTG TGAAGTGGTT CAGGTGGTG CAGAGGCGCT	4140
CATGGCCTCC TGCTTCTTGC CTACCACTTG GCCAGTGCCC ACCCAGCCCC TCAGGTGGCA	4200
CATCTGGAGG GCAGGGGTTG AGGGGCCACC ACCACACATG CCTTCTGTTG GTGAAGCCCT	4260
TTGGCTGCCC CACTCTCCTT GGATGGGTGT TGCTCCCTTA TCCCCAAATC ACTCTATACA	4320
TCCAATTCAG GAAACAAACA TGGTGGCAAT TCTACACAAA AAGAGATGAG ATTAACAGTG	4380
CAGGGTTGGG GTCTGCATTG GAGGTGCCCT ATAAACCAGA AGAGAAAATA CTGAAAGCAC	4440
AGGGGCAGGG ACAGACCAGA CCAGACCCAG GAGTCTCCAA AGCACAGAGT GGCAAACAAA	4500
ACCCGAGCTG AGCATCAGGA CCTTGCCTCG AATTGTCTTC CAGTATTACG GTGCCTCTTC	4560
TCTGCCCCCT TTCCCAGGGT ATCTGTGGGT TGCCAGGCTG GGGAGGGCAA CCATAGCCAC	4620
ACCACAGGAT TTCCTGAAAG TTTACAATGC AGTAGATTT TGGGGTGTAG GGTGGCAGCT	4680
CCCCAAGGCC CTGCCCCCA GCCCACCCA CTCATGACTC TAAGTGTGTT GTATTAATAT	4740
TTATTTATTT GGAGATGTTA TTTATTAGAT GATATTTATT GCAGAATTTT TATTCTTGTA	4800
TTAACAATA AAATGCTTGC CCCAGAACTT AGTCTCTTTG CCCAGCCTCA CCCCTCCTGG	4860
TGCTCATCAG ACTCTTGCCA CCCCTGGCTC CCACTCCCTG CTTGCCTCTG GTGGAGCTGC	4920
ACAGAGCTCT GGGAAGAGGC CCTCTTCTC CCCGCACTGG GGCGATGGGC GCACCTCAGA	4980
CTTACCACT GCTGCTGCCA CCACCAACCC CTTGATCCCT CAGTCCTCCC ACACAGCTTC	5040
TGTCCACCCC AGGTTTCCCT CACCCACCT TTGCTAAGTC TTCCTCA	5087

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 819 base pairs

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(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 95..769

(ix) FEATURE:  
 (A) NAME/KEY: mat\_peptide  
 (B) LOCATION: 176..769

(ix) FEATURE:  
 (A) NAME/KEY: sig\_peptide  
 (B) LOCATION: 95..175

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATTATTAAA GCTTCGCCGG AGCCGCGGCT CGCCCTCCCA CTCCGCCAGC CTCTGGGAGA	60
GGAGCCGCGC CCGGCCGGCC CGGCCCCAG CCCC ATG GAC CTC CGA GCA GGG	112
Met Asp Leu Arg Ala Gly	
-27 -25	
GAC TCG TGG GGG ATG TTA GCT TGC CTA TGC ACG GTG CTG TGG CAC CTC	160
Asp Ser Trp Gly Met Leu Ala Cys Leu Cys Thr Val Leu Trp His Leu	
-20 -15 -10	
CCT GCA GTG CCA GCT CTT AAT CGC ACA GGA GAT CCA GGC CCT GGC CCC	208
Pro Ala Val Pro Ala Leu Asn Arg Thr Gly Asp Pro Gly Pro Gly Pro	
-5 1 5 10	
TCC ATC CAG AAA ACC TAT GAC CTC ACC CGC TAC CTG GAG CAT CAA CTC	256
Ser Ile Gln Lys Thr Tyr Asp Leu Thr Arg Tyr Leu Glu His Gln Leu	
15 20 25	
CGC AGC TTA GCT GGG ACC TAC CTG AAC TAC CTG GGG CCC CCT TTC AAC	304
Arg Ser Leu Ala Gly Thr Tyr Leu Asn Tyr Leu Gly Pro Pro Phe Asn	
30 35 40	
GAG CCT GAC TTC AAT CCT CCT CGA CTG GGG GCA GAA ACT CTG CCC AGG	352
Glu Pro Asp Phe Asn Pro Pro Arg Leu Gly Ala Glu Thr Leu Pro Arg	
45 50 55	
GCC ACG GTC AAC TTG GAA GTG TGG CGA AGC CTC AAT GAC AGG CTG CGG	400
Ala Thr Val Asn Leu Glu Val Trp Arg Ser Leu Asn Asp Arg Leu Arg	
60 65 70 75	
CTG ACC CAG AAC TAT GAG GCG TAC AGT CAC CTC CTG TGT TAC TTG CGT	448
Leu Thr Gln Asn Tyr Glu Ala Tyr Ser His Leu Leu Cys Tyr Leu Arg	
80 85 90	

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GGC CTC AAC CGT CAG GCT GCC ACA GCT GAA CTC CGA CGT AGC CTG GCC	496
Gly Leu Asn Arg Gln Ala Ala Thr Ala Glu Leu Arg Arg Ser Leu Ala	
95 100 105	
CAC TTC TGT ACC AGC CTC CAG GGC CTG CTG GGC AGC ATT GCA GGT GTC	544
His Phe Cys Thr Ser Leu Gln Gly Leu Leu Gly Ser Ile Ala Gly Val	
110 115 120	
ATG GCG ACG CTT GGC TAC CCA CTG CCC CAG CCT CTG CCA GGG ACT GAG	592
Met Ala Thr Leu Gly Tyr Pro Leu Pro Gln Pro Leu Pro Gly Thr Glu	
125 130 135	
CCA GCC TGG GCC CCT GGC CCT GCC CAC AGT GAC TTC CTC CAG AAG ATG	640
Pro Ala Trp Ala Pro Gly Pro Ala His Ser Asp Phe Leu Gln Lys Met	
140 145 150 155	
GAT GAC TTC TGG CTG CTG AAG GAG CTG CAG ACC TGG CTA TGG CGT TCA	688
Asp Asp Phe Trp Leu Leu Lys Glu Leu Gln Thr Trp Leu Trp Arg Ser	
160 165 170	
GCC AAG GAC TTC AAC CGG CTT AAG AAG AAG ATG CAG CCT CCA GCA GCT	736
Ala Lys Asp Phe Asn Arg Leu Lys Lys Lys Met Gln Pro Pro Ala Ala	
175 180 185	
TCA GTC ACC CTG CAC TTG GAG GCA CAT GGT TTC TGACCTCTGA CCCTTAACCC	789
Ser Val Thr Leu His Leu Glu Ala His Gly Phe	
190 195	
CCACACCTCC AGGCCAGTC AGCTGTGCTT	819

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 225 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Asp Leu Arg Ala Gly Asp Ser Trp Gly Met Leu Ala Cys Leu Cys	
-27 -25 -20 -15	
Thr Val Leu Trp His Leu Pro Ala Val Pro Ala Leu Asn Arg Thr Gly	
-10 -5 1 5	
Asp Pro Gly Pro Gly Pro Ser Ile Gln Lys Thr Tyr Asp Leu Thr Arg	
10 15 20	
Tyr Leu Glu His Gln Leu Arg Ser Leu Ala Gly Thr Tyr Leu Asn Tyr	
25 30 35	
Leu Gly Pro Pro Phe Asn Glu Pro Asp Phe Asn Pro Pro Arg Leu Gly	
40 45 50	
Ala Glu Thr Leu Pro Arg Ala Thr Val Asn Leu Glu Val Trp Arg Ser	
55 60 65	
Leu Asn Asp Arg Leu Arg Leu Thr Gln Asn Tyr Glu Ala Tyr Ser His	
70 75 80 85	
Leu Leu Cys Tyr Leu Arg Gly Leu Asn Arg Gln Ala Ala Thr Ala Glu	

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90										95					100						
Leu	Arg	Arg	Ser	Leu	Ala	His	Phe	Cys	Thr	Ser	Leu	Gln	Gly	Leu	Leu						
			105					110					115								
Gly	Ser	Ile	Ala	Gly	Val	Met	Ala	Thr	Leu	Gly	Tyr	Pro	Leu	Pro	Gln						
		120					125					130									
Pro	Leu	Pro	Gly	Thr	Glu	Pro	Ala	Trp	Ala	Pro	Gly	Pro	Ala	His	Ser						
		135					140				145										
Asp	Phe	Leu	Gln	Lys	Met	Asp	Asp	Phe	Trp	Leu	Leu	Lys	Glu	Leu	Gln						
150					155					160					165						
Thr	Trp	Leu	Trp	Arg	Ser	Ala	Lys	Asp	Phe	Asn	Arg	Leu	Lys	Lys	Lys						
				170					175					180							
Met	Gln	Pro	Pro	Ala	Ala	Ser	Val	Thr	Leu	His	Leu	Glu	Ala	His	Gly						
			185					190					195								

Phe

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCAAGCTTC ACCATGGACC TCCGAGCAGG GGA CTC

36

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 64 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGCGGGGCGG CACTACTTGC ATCGTCGCGT CCTTGTACTC GAAGCCATGA GCGGGGAGGT  
GCAG

60

64

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 199 amino acids
  - (B) TYPE: amino acid

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(C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:  
 (A) NAME/KEY: Protein  
 (B) LOCATION: 1..178

(ix) FEATURE:  
 (A) NAME/KEY: Region  
 (B) LOCATION: -21..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

Met Asn Cys Val Cys Arg Leu Val Leu Val Val Leu Ser Leu Trp Pro
-20                      -15                      -10

Asp Thr Ala Val Ala Pro Gly Pro Pro Pro Gly Pro Pro Arg Val Ser
-5                      1                      5                      10

Pro Asp Pro Arg Ala Glu Leu Asp Ser Thr Val Leu Leu Thr Arg Ser
15                      20                      25

Leu Leu Ala Asp Thr Arg Gln Leu Ala Ala Gln Leu Arg Asp Lys Phe
30                      35                      40

Pro Ala Asp Gly Asp His Asn Leu Asp Ser Leu Pro Thr Leu Ala Met
45                      50                      55

Ser Ala Gly Ala Leu Gly Ala Leu Gln Leu Pro Gly Val Leu Thr Arg
60                      65                      70                      75

Leu Arg Ala Asp Leu Leu Ser Tyr Leu Arg His Val Gln Trp Leu Arg
80                      85                      90

Arg Ala Gly Gly Ser Ser Leu Lys Thr Leu Glu Pro Glu Leu Gly Thr
95                      100                      105

Leu Gln Ala Arg Leu Asp Arg Leu Leu Arg Arg Leu Gln Leu Leu Met
110                      115                      120

Ser Arg Leu Ala Leu Pro Gln Pro Pro Pro Asp Pro Pro Ala Pro Pro
125                      130                      135

Leu Ala Pro Pro Ser Ser Ala Trp Gly Gly Ile Arg Ala Ala His Ala
140                      145                      150                      155

Ile Leu Gly Gly Leu His Leu Thr Leu Asp Trp Ala Val Arg Gly Leu
160                      165                      170

Leu Leu Leu Lys Thr Arg Leu
175

```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 212 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: Protein  
(B) LOCATION: 1..182

(ix) FEATURE:

(A) NAME/KEY: Region  
(B) LOCATION: -30..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

Met Asn Ser Phe Ser Thr Ser Ala Phe Gly Pro Val Ala Phe Ser Leu
-30          -25          -20          -15

Gly Leu Leu Leu Val Leu Pro Ala Ala Phe Pro Ala Pro Val Pro Pro
          -10          -5          1

Gly Glu Asp Ser Lys Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr
      5          10          15

Ser Ser Glu Arg Ile Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile
  20          25          30

Ser Ala Leu Arg Lys Glu Thr Cys Asn Lys Ser Asn Met Cys Glu Ser
  35          40          45          50

Ser Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala
          55          60          65

Glu Lys Asp Gly Cys Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu
          70          75          80

Val Lys Ile Ile Thr Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr
          85          90          95

Leu Gln Asn Arg Phe Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln
  100          105          110

Met Ser Thr Lys Val Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn
  115          120          125          130

Leu Asp Ala Ile Thr Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu
          135          140          145

Thr Lys Leu Gln Ala Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His
          150          155          160

Leu Ile Leu Arg Ser Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala
          165          170          175

Leu Arg Gln Met
          180

```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 204 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: Protein

(B) LOCATION: 1..174

(ix) FEATURE:

(A) NAME/KEY: Region

(B) LOCATION: -30..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

Met Ala Gly Pro Ala Thr Gln Ser Pro Met Lys Leu Met Ala Leu Gln
-30          -25          -20          -15

Leu Leu Leu Trp His Ser Ala Leu Trp Thr Val Gln Glu Ala Thr Pro
          -10          -5          1

Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu
          5          10          15

Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys
          20          25          30

Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Leu
          35          40          45          50

Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
          55          60          65

Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu
          70          75          80

Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu
          85          90          95

Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala
          100          105          110

Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu
          115          120          125          130

Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg
          135          140          145

Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu
          150          155          160

Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
          165          170

```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 201 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Met Ser Arg Arg Glu Gly Ser Leu Glu Asp Pro Gln Thr Asp Ser Ser
 1           5           10           15
Val Ser Leu Leu Pro His Leu Glu Ala Lys Ile Arg Gln Thr His Ser
          20           25           30
Leu Ala His Leu Leu Thr Lys Tyr Ala Glu Gln Leu Leu Gln Glu Tyr
          35           40           45
Val Gln Leu Gln Gly Asp Pro Phe Gly Leu Pro Ser Phe Ser Pro Pro
          50           55           60
Arg Leu Pro Val Ala Gly Leu Ser Ala Pro Ala Pro Ser His Ala Gly
65           70           75           80
Leu Pro Val His Glu Arg Leu Arg Leu Asp Ala Ala Ala Leu Ala Ala
          85           90           95
Leu Pro Pro Leu Leu Asp Ala Val Cys Arg Arg Gln Ala Glu Leu Asn
          100          105          110
Pro Arg Ala Pro Arg Leu Leu Arg Arg Leu Glu Asp Ala Ala Arg Gln
          115          120          125
Ala Arg Ala Leu Gly Ala Ala Val Glu Ala Leu Leu Ala Ala Leu Gly
          130          135          140
Ala Ala Asn Arg Gly Pro Arg Ala Glu Pro Pro Ala Ala Thr Ala Ser
          145          150          155          160
Ala Ala Ser Ala Thr Gly Val Phe Pro Ala Lys Val Leu Gly Leu Arg
          165          170          175
Val Cys Gly Leu Tyr Arg Glu Trp Leu Ser Arg Thr Glu Gly Asp Leu
          180          185          190
Gly Gln Leu Leu Pro Gly Gly Ser Ala
          195          200

```

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 199 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Met Ala Phe Thr Glu His Pro Leu Thr Pro His Arg Arg Asp Leu Cys
 1           5           10           15
Ser Arg Ser Ile Trp Leu Ala Arg Lys Ile Arg Ser Asp Leu Thr Ala
          20           25           30

```

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```

Leu Thr Glu Ser Tyr Val Lys His Gln Gly Leu Asn Lys Asn Ile Asn
    35                      40                      45
Leu Asp Ser Ala Asp Gly Met Pro Val Ala Ser Thr Asp Gln Trp Ser
    50                      55                      60
Glu Leu Thr Glu Ala Glu Arg Leu Gln Glu Asn Leu Gln Ala Tyr Arg
    65                      70                      75                      80
Thr Phe His Val Leu Leu Ala Arg Leu Leu Glu Asp Gln Gln Val His
                      85                      90                      95
Phe Thr Pro Thr Glu Gly Asp Phe His Gln Ala Ile His Thr Leu Leu
                      100                      105                      110
Leu Gln Val Ala Ala Phe Ala Tyr Gln Ile Glu Glu Leu Met Ile Leu
                      115                      120                      125
Leu Glu Tyr Lys Ile Pro Arg Asn Glu Ala Asp Gly Met Pro Ile Asn
                      130                      135                      140
Val Gly Asp Gly Gly Leu Phe Glu Lys Lys Leu Trp Gly Leu Lys Val
                      145                      150                      155                      160
Leu Gln Glu Leu Ser Gln Trp Thr Val Arg Ser Ile His Asp Leu Arg
                      165                      170                      175
Phe Ile Ser Ser His Gln Thr Gly Ile Pro Ala Arg Gly Ser His Tyr
                      180                      185                      190
Ile Ala Asn Asn Lys Lys Met
                      195

```

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 252 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..227

## (ix) FEATURE:

- (A) NAME/KEY: Region
- (B) LOCATION: -25..0

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

Met Gly Val Leu Leu Thr Gln Arg Thr Leu Ser Leu Val Leu Ala
-25                      -20                      -15                      -10
Leu Leu Phe Pro Ser Met Ala Ser Met Ala Ala Ile Gly Ser Cys Ser
                      -5                      1                      5
Lys Glu Tyr Arg Val Leu Leu Gly Gln Leu Gln Lys Gln Thr Asp Leu
    10                      15                      20

```

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```

Met Gln Asp Thr Ser Arg Leu Leu Asp Pro Tyr Ile Arg Ile Gln Gly
 25                               30                35

Leu Asp Val Pro Lys Leu Arg Glu His Cys Arg Glu Arg Pro Gly Ala
 40                               45                50                55

Phe Pro Ser Glu Glu Thr Leu Arg Gly Leu Gly Arg Arg Gly Phe Leu
                               60                65                70

Gln Thr Leu Asn Ala Thr Leu Gly Cys Val Leu His Arg Leu Ala Asp
                               75                80                85

Leu Glu Gln Arg Leu Pro Lys Ala Gln Asp Leu Glu Arg Ser Gly Leu
                               90                95                100

Asn Ile Glu Asp Leu Glu Lys Leu Gln Met Ala Arg Pro Asn Ile Leu
 105                               110                115

Gly Leu Arg Asn Asn Ile Tyr Cys Met Ala Gln Leu Leu Asp Asn Ser
 120                               125                130                135

Asp Thr Ala Glu Pro Thr Lys Ala Gly Arg Gly Ala Ser Gln Pro Pro
                               140                145                150

Thr Pro Thr Pro Ala Ser Asp Ala Phe Gln Arg Lys Leu Glu Gly Cys
                               155                160                165

Arg Phe Leu His Gly Tyr His Arg Phe Met His Ser Val Gly Arg Val
 170                               175                180

Phe Ser Lys Trp Gly Glu Ser Pro Asn Arg Ser Arg Arg His Ser Pro
 185                               190                195

His Gln Ala Leu Arg Lys Gly Val Arg Arg Thr Arg Pro Ser Arg Lys
 200                               205                210                215

Gly Lys Arg Leu Met Thr Arg Gly Gln Leu Pro Arg
                               220                225

```

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 202 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (ix) FEATURE:  
 (A) NAME/KEY: Protein  
 (B) LOCATION: 1..180

- (ix) FEATURE:  
 (A) NAME/KEY: Region  
 (B) LOCATION: -22..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

Met Lys Val Leu Ala Ala Gly Val Val Pro Leu Leu Leu Val Leu His
  -20                               -15                -10

```

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Trp Lys His Gly Ala Gly Ser Pro Leu Pro Ile Thr Pro Val Asn Ala  
     -5                                  1                                  5                                  10  
 Thr Cys Ala Ile Arg His Pro Cys His Asn Asn Leu Met Asn Gln Ile  
                                   15                                  20                                  25  
 Arg Ser Gln Leu Ala Gln Leu Asn Gly Ser Ala Asn Ala Leu Phe Ile  
                                   30                                  35                                  40  
 Leu Tyr Tyr Thr Ala Gln Gly Glu Pro Phe Pro Asn Asn Leu Asp Lys  
                                   45                                  50                                  55  
 Leu Cys Gly Pro Asn Val Thr Asp Phe Pro Pro Phe His Ala Asn Gly  
                                   60                                  65                                  70  
 Thr Glu Lys Ala Lys Leu Val Glu Leu Tyr Arg Ile Val Val Tyr Leu  
     75                                  80                                  85                                  90  
 Gly Thr Ser Leu Gly Asn Ile Thr Arg Asp Gln Lys Ile Leu Asn Pro  
                                   95                                  100                                  105  
 Ser Ala Leu Ser Leu His Ser Lys Leu Asn Ala Thr Ala Asp Ile Leu  
                                   110                                  115                                  120  
 Arg Gly Leu Leu Ser Asn Val Leu Cys Arg Leu Cys Ser Lys Tyr His  
                                   125                                  130                                  135  
 Val Gly His Val Asp Val Thr Tyr Gly Pro Asp Thr Ser Gly Lys Asp  
     140                                  145                                  150  
 Val Phe Gln Lys Lys Lys Leu Gly Cys Gln Leu Leu Gly Lys Tyr Lys  
     155                                  160                                  165                                  170  
 Gln Ile Ile Ala Val Leu Ala Gln Ala Phe  
                                   175                                  180

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGCGCTACGG TCGACCCGGC GTTTTTTTTT TTTTTTTTTT TTACG

45

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGAAGGAAAA AAGCGGCCGC TACA

24

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## I CLAIM:

1. A nucleic acid molecule encoding a  
5 polypeptide selected from the group consisting of:  
    (a) the nucleic acid molecule of SEQ ID NO:1;  
    (b) the nucleic acid molecule of SEQ ID NO:3;  
    (c) a nucleic acid molecule encoding the  
polypeptide of SEQ ID NO:2 or a biologically active  
10 fragment thereof;  
    (d) a nucleic acid molecule that encodes a  
polypeptide that is at least 70 percent identical to  
the polypeptide of SEQ ID NO:2;  
    (e) a nucleic acid molecule that hybridizes  
15 under stringent conditions to any of (a)-(d) above; and  
    (f) a nucleic acid molecule that is the  
complement of any of (a)-(e) above.
2. A nucleic acid molecule encoding a  
20 polypeptide selected from the group consisting of:  
    (a') the nucleic acid molecule of SEQ ID  
NO:4;  
    (b') a nucleic acid molecule encoding the  
polypeptide of SEQ ID NO:5 or a biologically active  
25 fragment thereof;  
    (c') a nucleic acid molecule that encodes a  
polypeptide that is at least 70 percent identical to  
the polypeptide of SEQ ID NO:5;  
    (d') a nucleic acid molecule that hybridizes  
30 under stringent conditions to any of (a')-(c') above;  
and  
    (e') a nucleic acid molecule that is the  
complement of any of (a')-(d') above.
- 35 3. The nucleic acid molecule that is SEQ ID  
NO:1.

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4. The nucleic acid molecule that is SEQ ID NO:3.
- 5 5. A nucleic acid molecule encoding the polypeptide of SEQ ID NO:2.
6. A nucleic acid molecule encoding amino acids 1-198 of SEQ ID NO:2
- 10 7. A vector comprising a nucleic acid molecule of any of claims 1 to 6.
8. A host cell comprising a vector of claim
- 15 7.
9. A process for producing an NNT-1 polypeptide comprising the steps of:
- (a) expressing a polypeptide encoded by a
- 20 nucleic acid of any of claims 1-6 in a suitable host; and
- (b) isolating the polypeptide.
10. An NNT-1 polypeptide selected from the
- 25 group consisting of:
- (a) the polypeptide of SEQ ID NO:2;
- (b) the polypeptide that is amino acids 1-198 of SEQ ID NO:2;
- (c) a polypeptide that is at least 70 percent
- 30 identical to the polypeptide of (a) or (b); and
- (d) a biologically active fragment of any of (a) - (c).
11. An NNT-1 polypeptide selected from the
- 35 group consisting of:

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- (a') the polypeptide of SEQ ID NO:5;  
(b') the polypeptide that is amino acids 1-198 of SEQ ID NO:5;  
(c') a polypeptide that is at least 70 percent identical to the polypeptide of (a') or (b');  
and  
(d') a biologically active fragment of any of (a') - (c').

10           12. An NNT-1 polypeptide that is the polypeptide of SEQ ID NO:2 or a biologically active fragment thereof.

15           13. An NNT-1 polypeptide that is the polypeptide of SEQ ID NO:5 or a biologically active fragment thereof.

20           14. The NNT-1 polypeptide of claim 12 or 13 that does not possess an amino terminal methionine.

            15. The NNT-1 polypeptide of claim 12 or 13 that additionally possesses an amino terminal methionine.

25           16. An antibody or fragment thereof which specifically binds human NNT-1..

            17. The antibody of claim 16 that is a monoclonal antibody.

30           18. A method of treating a patient suffering from a neurological or immunological disease or disorder, comprising administering to said patient an effective amount of an NNT-1 polypeptide according to  
35   any of Claims 12-15.

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19. A method according to Claim 18, wherein  
said disease or disorder is selected from Alzheimer's  
disease, Parkinson's disease, amyotrophic lateral  
5 sclerosis, Charcot-Marie-Tooth syndrome, Huntington's  
disease, peripheral neuropathy, dystrophy, or  
degeneration of the neural retina.

20. A method according to Claim 18, wherein  
10 said disease or disorder is characterized by a  
deficiency of B-cells or T cells.

21. A method according to Claim 20, wherein  
said disease or disorder is common variable  
15 immunodeficiency (CVID), selective IgA deficiency,  
hypogammaglobulinemia, and X-linked  
agammaglobulinemia.

22. A method of boosting immunoreactivity  
20 and antibody production upon vaccination, comprising  
administering to a patient in need thereof an effective  
amount of an NNT-1 polypeptide according to any of  
Claims 12-15.

23. A method of treating an inflammatory  
25 condition in a patient in need thereof, comprising  
administering to said patient an effective amount of  
NNT-1 polypeptide according to any of Claims 12-15.

30

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## FIG. 1

1 ATTAAGCTT CGCCGGAGCC GCGGCTCGCC CTCCCACTCC GCCAGCCTCC  
51 GGGAGAGGAG CCGCACCCGG CCGGCCCAGC CCCAGCCCCA TGGACCTCCG  
101 AGCAGGGGAC TCGTGGGGGA TGTTAGCGTG CCTGTGCACG GTGCTCTGGC  
151 ACCTCCCTGC AGTGCCAGCT CTCAATCGCA CAGGGGACCC AGGGCCTGGC  
201 CCCTCCATCC AGAAAACCTA TGACCTCACC CGCTACCTGG AGCACCAACT  
251 CCGCAGCTTG GCTGGGACCT ATCTGAACTA CCTGGGCCCC CCTTTCAACG  
301 AGCCAGACTT CAACCCTCCC CGCCTGGGGG CAGAGACTCT GCCCAGGGCC  
351 ACTGTTGACT TGGAGGTGTG GCGAAGCCTC AATGACAAAC TGGCGCTGAC  
401 CCAGAACTAC GAGGCCTACA GCCACCTTCT GTGTTACTTG CGTGGCCTCA  
451 ACCGTCAGGC TGCCACTGCT GAGCTGCGCC GCAGCCTGGC CCACTTCTGC  
501 ACCAGCCTCC AGGGCCTGCT GGCAGCATT GCGGGCGTCA TGGCAGCTCT  
551 GGGCTACCCA CTGCCCCAGC CGCTGCCTGG GACTGAACCC ACTTGGAATC  
601 CTGGCCCTGC CCACAGTGAC TTCCTCCAGA AGATGGACGA CTTCTGGCTG  
651 CTGAAGGAGC TGCAGACCTG GCTGTGGCGC TCGGCCAAGG ACTTCAACCG  
701 GCTCAAGAAG AAGATGCAGC CTCCAGCAGC TGCAGTCACC CTGCACCTGG  
751 GGGCTCATGG CTTCTGACTT CTGACCTTCT CCTCTTCGCT CCCCCC

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## FIG.2

Genomic sequences of the human NNT-1

```
1  aacctgcgag tgggcctggc ggatgggatt attaaagctt cgccggagcc
51  gcggtctgcc ctcccactcc gccagcctcc gggagaggag ccgcacccgg
101 ccggcccagc cccagcccccA TGGACCTCCG AGCAGgt--- -----
      ----- ( >1 kb ) ----- tgaaaaccca

151 aactagccct gctcttcata acatgacaag cagcgcccca tctgatacct
201 aaaccgacca agtcacagcc ctccaactca ccctctgcct gccagacct
251 caccacatcc ttgstggact caaacctcaa ccgcactaaa tcaaccaaat
301 cccaagtcta aactaatctg aaacttttaa agtaaccag tccttaaacc
351 taacctagcc caatgccaat tataatctacc ctagccaaac cctaactgcc
401 tttgccagtc caaagtgtcc actgaatcct caccttggtc ctactgaaa
451 atcccagaaa agcatatttc ccactgccc acatccctcc ttacagcacc
501 caaccctggc ctctggactc ctggtatcct gggatgtcca aactctgcag
551 tgccatcagc caacaagccc gactcgtcaa atgcacctct ctcccttcct
601 gtccccaccc ttgcaggctg atggaaaggc ctattgaag tccaactttt
651 cccacactaa caccaagaac ggggtgaacc tccacactgc caccgttccc
```

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## FIG.2A

701 tgagagtgag cactaaatct ccttcaatct aacccccacc tacacttccc  
751 aactcagga atcacatcct agaataacc caaaactaag ccccataagg  
801 cagcccgacc ctagtggtct aaccctatac cttgcttctt atgggtgagt  
851 ctgttcttgg cggcgcctc tctcctgctt cctcccttag agctgactgt  
901 gctcagcctg ccagctctga catgtgctgt ctcccaccct ctgactcccc  
951 tcaagctgca gtgggactgg aagactggca ggaagctagg gtacaactgg  
1001 aacacaggca ggtcgacctg cagtccctag gcctggcccc gtccctccat  
1051 gtacacacat atacatgttg gcacacacac agtggcacac atgccaaaga  
1101 ctctctcagc tgacacacag atccattctc aagtatctac tgatagacac  
1151 tcatgcgtgc caagtcctca tcctcaaaca tacacatgcc tctctttctc  
1201 tcccgctcttg ccaggagtgt tccccctcct ccateccctc tgccctccat  
1251 ctgggtgtccc accctcacc cccaccacgc ccaagggtggg gacagacacc  
1301 tgaggggctg ccagctgctt ccccggtgtgg gcccgggccg cgctcatgct  
1351 tctcgtccat cctgcccaca gGGGACTCGT GGGGGATGTT AGCGTGCCTG  
1401 TGCACGGTGC TCTGGCACCT CCCTGCAGTG CCAGCTCTCA ATCGCACAGG  
1451 GGACCCAGGG CCTGGCCCCCT CCATCCAGAA AACCTATGAC CTCACCCGCT  
1501 ACCTGGAGCA CCAACTCCGC AGCTTGGCTG GGACCTATgt gagtatccag  
1551 cgtaggaatc tgggagttgg ggaggagtga ggagttgggg aaagacagtc  
1601 ctaaccgtgg agggttctgg taaatgatgg ggtgaggagg ggctctttgg

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## FIG.2B

1651 ctcccaccag tccccctgtc tgggtctatct cctgcccttc cctcttaggt  
1701 ggccccccca cttccccatc cctggcccca ggactaggca tgtgggcagg  
1751 cctcgcaccc gccttggccc attgccccac tggctgccag cccagccgcc  
1801 cgcttcccc tggggggcgg ggaagtctcc tctgtttaca ccgtgttgtg  
1851 gtgtctcttg cgcggggcggg gttgggtggg gacagagggg cccacctcc  
1901 catgcctgcg ttccagctcg cctctgcccc cagacctggg gccctgctgc  
1951 tctggacca ggggcctccc ttccgtctgc ctctcccatc ctagctgggc  
2001 ctccatagggg ggtcatgggg gaaggggact gtagggaacc caggcagtag  
2051 tggcagggggg tttaggggtgt ggatggaggt tatgctgtaa ggatttgggg  
2101 gtggtccaga ggtgttcaga gagcccagga gagaaggaag gagggttgga  
2151 ggagccgagg caccatgggg aaccggcccc ctcttcccgt gtccctcttc  
2201 cacatcccag accctactct ggagccaggg aaagaaaagg gaagaagggtg  
2251 gcgggggagc tggctccagc cccaggatac accgaggaaa ttagtttgtc  
2301 tctgtgcttg tcagcgtgtg aacctcccc tgggcccttg cctatcccag  
2351 gcctctcccc ttgcttctcc cttctttccc agttatacat ctccctcatc  
2401 cctttccctg ggccccagcc gctccccga gggttggaaa gggctctgcc  
2451 ctcttcccta taccatgctg tcttccatag ccttctctct gtccctactca  
2501 tgagactgcc tccatttctt ccttctgcaa cctgctcct atcagctgaa

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## FIG.2C

2551 cccttctttc ggagtgttag tgagtacccg tctctcccca gcccctcagc  
2601 tgggtgggcct ggggtgtgtca gcggaacatg gggctctggg tccaatgggc  
2651 cactctcatc tctctcttgt tccttgtgca gaaaaccttt gcttcactcc  
2701 actgccctct ctagttcccg accctttttc tctcctgggt ttccctgcca  
2751 aatttctcca aggagtgggc tacacctct gcctccactt cctctccacc  
2801 cactcacttc ttaacccct gcaatctggc ttccaggccc cagcaatggg  
2851 tctctccaag gtcgtcaggc acctccttgc caagcccgac agtggtttga  
2901 aggctcatc tccttgctgt ctgttttgca gccacactgc tgagcgctgc  
2951 tgccttctcg aactcctctt ccttgggtctc tgcactctcc tggggcacct  
3001 tctacctctc cagctcctcc aggctcctct tcctctctgt cctgccccca  
3051 cagcggggcac tctcccaagg tttgcccacc cagccaatca gcacgtcctt  
3101 cctgagcgtc ttgtgcgtct cctcctctc ctttttctac gcctctccat  
3151 tggagagctc accaccgcca ctgcttcaac tgtcacctgc atacaaatga  
3201 tatecttatt ggaaaaactc agggaggcca tgaacaaaga agcctagcat  
3251 ggagacaggg ccagtgtcag gggacacaaa aaatagaaac tttgggagca  
3301 ggtatctcct tgggtgggtgag ccagcgggtc tgccctctc cttccccatc  
3351 acctctcct ttccacagCT GAACTACCTG GGCCCCCTT TCAACGAGCC  
3401 AGACTTCAAC CCTCCCCGCC TGGGGGCAGA GACTCTGCCC AGGGCCACTG  
3451 TTGACTTGGA GGTGTGGCGA AGCCTCAATG ACAAAGTGGC GCTGACCCAG

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## FIG.2D

3501 AACTACGAGG CCTACAGCCA CCTTCTGTGT TACTTGCGTG GCCTCAACCG  
3551 TCAGGCTGCC ACTGCTGAGC TGCGCCGCAG CCTGGCCCAC TTCTGCACCA  
3601 GCCTCCAGGG CCTGCTGGGC AGCATTTGCGG GCGTCATGGC AGCTCTGGGC  
3651 TACCCACTGC CCCAGCCGCT GCCTGGGACT GAACCCACTT GGACTCCTGG  
3701 CCCTGCCCAC AGTGACTTCC TCCAGAAGAT GGACGACTTC TGGCTGCTGA  
3751 AGGAGCTGCA GACCTGGCTG TGGCGCTCGG CCAAGGACTT CAACCGGCTC  
3801 AAGAAGAAGA TGCAGCCTCC AGCAGCTGCA GTCACCCTGC ACCTGGGGGC  
3851 TCATGGCTTC tgacttctga ctttctcctc ttctgtcccc cttcaaacc  
3901 tgctcccact ttgtgagagc cagccctgta tgccaacacc tgttgagcca  
3951 ggagacagaa gctgtgagcc tctggccctt tcctggaccg gctgggcggtg  
4001 tgatgcgatc agccctgtct cctccccacc tcccaaaggt ctaccgagct  
4051 ggggaggagg tacagtaggc cctgtcctgt cctgtttcta caggaagtca  
4101 tgctcgaggg agtgtgaagt ggttcagggt ggtgcagagg cgctcatggc  
4151 ctcttgcttc ttgcctacca cttggccagt gccaccacag cccctcaggt  
4201 ggcacatctg gagggcaggg gttgaggggc caccaccaca catgcctttc  
4251 tgggggtgaag ccctttggct gcccactct ccttgatgg gtgttgctcc

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## FIG.2E

4301 cttatcccca aatcactcta tacatccaat tcaggaaaca aacatggttg  
4351 caattctaca caaaaagaga tgagattaac agtgcagggt tggggtctgc  
4401 attggaggtg ccctataaac cagaagagaa aatactgaaa gcacaggggc  
4451 agggacagac cagaccagac ccaggagtct ccaaagcaca gagtggcaaa  
4501 caaaacccga gctgagcatc aggaccttgc ctggaattgt cttccagtat  
4551 tacggtgcct cttctctgcc ccctttccca gggatatctgt gggttgccag  
4601 gctggggagg gcaaccatag ccacaccaca ggatttctctg aaagtttaca  
4651 atgcagtagc attttgggggt gtaggggtggc agctcccca ggccttgccc  
4701 cccagcccca ccactcatg actctaagtg tgttgtatta atatttattt  
4751 atttggagat gttatttatt agatgatatt tattgcagaa tttctattct  
4801 tgtattaaca aataaaatgc ttgccccaga acttagtctc ttgcccagc  
4851 ctcacccctc ctggtgctca tcagactctt gccacccctg gctcccactc  
4901 cctgcttgcc tctggtggag ctgcacagag ctctgggaag aggccctctt  
4951 cctccccgca ctggggcgat gggcgcacct cagacttacc cactgctgct  
5001 gccaccacca accccttgat ccctcagtcc tcccacacag cttctgtcca  
5051 cccaggttt ccctacccc acctttgcta agtcttcctc a

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## FIG.3

-27 1  
MDLR AGDSWGMLAC LCTVLWHLPA VPALNRTGDP GPGPSIQKTY 17

DLTRYLEHQL RSLAGTYLNY LGPPFNEPDF NPPRLGAETL PRATVDLEW 67

RSINDKLRLT QNYEAYSHLL CYLRGLNRQA ATAELRRSLA HFCTSLQGLL 117

GSIAGVMAAL GYPLPQPLPG TEPTWTPGPA HSDFLQKMDD FWLLKELQW 167

198  
LWRSKDFNR LKKKMPPAA AVTLHLGAHG F\* 198

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## FIG.4

1 TATTATTAAA GCTTCGCCGG AGCCGCGGCT CGCCCTCCCA CTCCGCCAGC  
51 CTCTGGGAGA GGAGCCGCGC CCGGCCGGCC CGGCCCCCAG CCCCATGGAC  
101 CTCCGAGCAG GGGACTCGTG GGGGATGTTA GCTTGCCTAT GCACGGTGCT  
151 GTGGCACCTC CCTGCAGTGC CAGCTCTTAA TCGCACAGGA GATCCAGGCC  
201 CTGGCCCCCTC CATCCAGAAA ACCTATGACC TCACCCGCTA CCTGGAGCAT  
251 CAACTCCGCA GCTTAGCTGG GACCTACCTG AACTACCTGG GGCCCCCTTT  
301 CAACGAGCCT GACTTCAATC CTCCTCGACT GGGGGCAGAA ACTCTGCCCA  
351 GGGCCACGGT CAACTTGGA GTGTGGCGAA GCCTCAATGA CAGGCTGCGG  
401 CTGACCCAGA ACTATGAGGC GTACAGTCAC CTCCTGTGTT ACTTGCGTGG  
451 CCTCAACCGT CAGGCTGCCA CAGCTGAACT CCGACGTAGC CTGGCCCCACT  
501 TCTGTACCAG CCTCCAGGGC CTGCTGGGCA GCATTGCAGG TGTCATGGCG  
551 ACGCTTGGCT ACCCACTGCC CCAGCCTCTG CCAGGGACTG AGCCAGCCTG  
601 GGCCCCCTGGC CCTGCCCACA GTGACTTCCT CCAGAAGATG GATGACTTCT  
651 GGCTGCTGAA GGAGCTGCAG ACCTGGCTAT GGC GTTCAGC CAAGGACTTC  
701 AACCGGCTTA AGAAGAAGAT GCAGCCTCCA GCAGCTTCAG TCACCCTGCA  
751 CTTGGAGGCA CATGGTTTCT GACCTCTGAC CCTTAACCCC CACACCTCCA  
801 GGCCCAGTCA GCTGTGCTT

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## FIG.5

-27 1 23  
MDLRAGDSWG MLACLCTVLW HLPVAPALNR TGDPGPGPSI QKTYDLTRYL  
EHQLRSLAGT YLNYLGPPFN EPDFNPPRLG AETLPRATVN LEVWRS LNDR 73  
LRLTQNYEAY SHLLCYLRGL NRQAATAELR RSLAHFCTSL QGLLGS IAGV 123  
MATLGYPLPQ PLPGTEPAWA PGPAHSDFLQ KMDDFWLLKE LQTWLWRS AK 173  
198  
DFNRLKKKMQ PPAASVTLHL EAHGF\* 198

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## FIG.6

	1				50
NNT-1	.....	.....	MDL	RAGDSWGMLA	CLCTVLWHLP
Il-11	.....	.....	.....	MNCVCRLVLV	VLS..LWPDT
Il-6	.....	.....	MNSFSTSAF	GPVAFSLGLL	LVLPAAFPAP
GCSF	.....	.....	.....	MAGPAT	QSPMKLMALQ
Cardiotrophin	.....	.....	.....	.....	LLL...WWSA
CNTF	.....	.....	.....	.....	MSRREG
Oncostatin	.....	.....	.....	..MGVLLTQR	....MAFTEH
LIF	.....	.....	.....	MKVLAAGVVP	TLLSLVLALL
					LLLVLHWKHG
	51				100
NNT-1	AVPALNRTG.	...DPGPGPS	IQKTYDLTRY	LEHQLRSLAG	TYLNYLGPPF
Il-11	AVAPGPPPGP	PRVSPDPRAE	LDSTVLLTRS	LLADTRQLAA	QLRDKFPA..
Il-6	VPPGEDSKDV	AAPHRQPLTS	SERIDKQIRY	ILDGISALRK	ETCN.....
GCSF	LWTVQEATPL	GPASSLPQSF	LLKCLEQVRK	IQGDGAALQE	KLCA.....
Cardiotrophin	SLEDPQTDSS	VSLLPHEAK	IRQTHSLAHL	LTKYAEQLLQ	EYVQLQGDPF
CNTF	S.....	.PLTPHRRDL	CSRSIWLARK	IRSDLTALTE	SYVKHQGLNK
Oncostatin	FPSMASMAAI	GSCSKEYRVL	LGQLQKQTD.	LMQDTSRLLD	PYIRIQGLDV
LIF	AGSPLPITPV	NATCAIRHPC	HNNLMNQIRS	QLAQLNGSAN	AL.....
	101				150
NNT-1	NEPDFNPPRL	GAETLPRATV	DLEVWRSLND	KLRLTQN..Y	EAY..SHLLCY
Il-11	.DGDHNLDSL	PTLAMSAGAL	GALQLPGVLT	RLR.....	....ADLLSY
Il-6	...KSNMCES	SKEALAENNL	NLPKMAEKDG	CFQSGFN..E	ETCLVKIITG
GCSF	...TYKLCHP	EELVLLGHSL	GIPW.APLSS	CPSQALQ..L	AGCLSQHLHG
Cardiotrophin	...GLPSFSP	PRLPVAGLSA	PAPSHAGLPV	HERLRD..A	AALAALPPLL
CNTF	...NINLDSA	DGMPVAS...	.TDQWSELTE	AERLQEN..L	QAYRTFHVLL
Oncostatin	PKLREHCRER	PGAFPSEETL	RLGRRGFLQ	TLNATLGCVL	HRLADLEQRL
LIF	....FILYYT	AQGEPPFNNL	DKLCGPVNTD	FPPFHANGTE	KAKLVELYRI
	151				200
NNT-1	LRGLN..RQA	ATAELR...R	SLAHFCTSLQ	GLLGSIAQVM	AAL..GYP.L
Il-11	LRHVQWLRRA	GGSSLKTLEP	ELGTLQARLD	RLRRLQLLM	SRL..ALP.Q
Il-6	LLEFEVYLEY	LQNRFESSEE	QARAVQMSTK	VLIQFLQKKA	KNL..DAI.T
GCSF	LFLYQGLLQA	LEGISPELGP	TLDTLQLDVA	DFATTIWQQM	EEL..GMA.P
Cardiotrophin	D.AVCRRQAE	LNPRAPRLR	RLEDAARQAR	ALGAAVEALL	AAL..GAANR
CNTF	ARLLEDQQVH	FTPTEGDFHQ	AIHTLLQVA	AFAYQIEELM	ILL..E..YK
Oncostatin	PKAQDLERSG	LNIEDLEKLQ	MARPNILGLR	NNIYCMQALL	DNS..DTAEP
LIF	VVYLGTSLGN	ITRDQKILNP	SALSLHSLN	ATADILRGLL	SNVLCRLCSK

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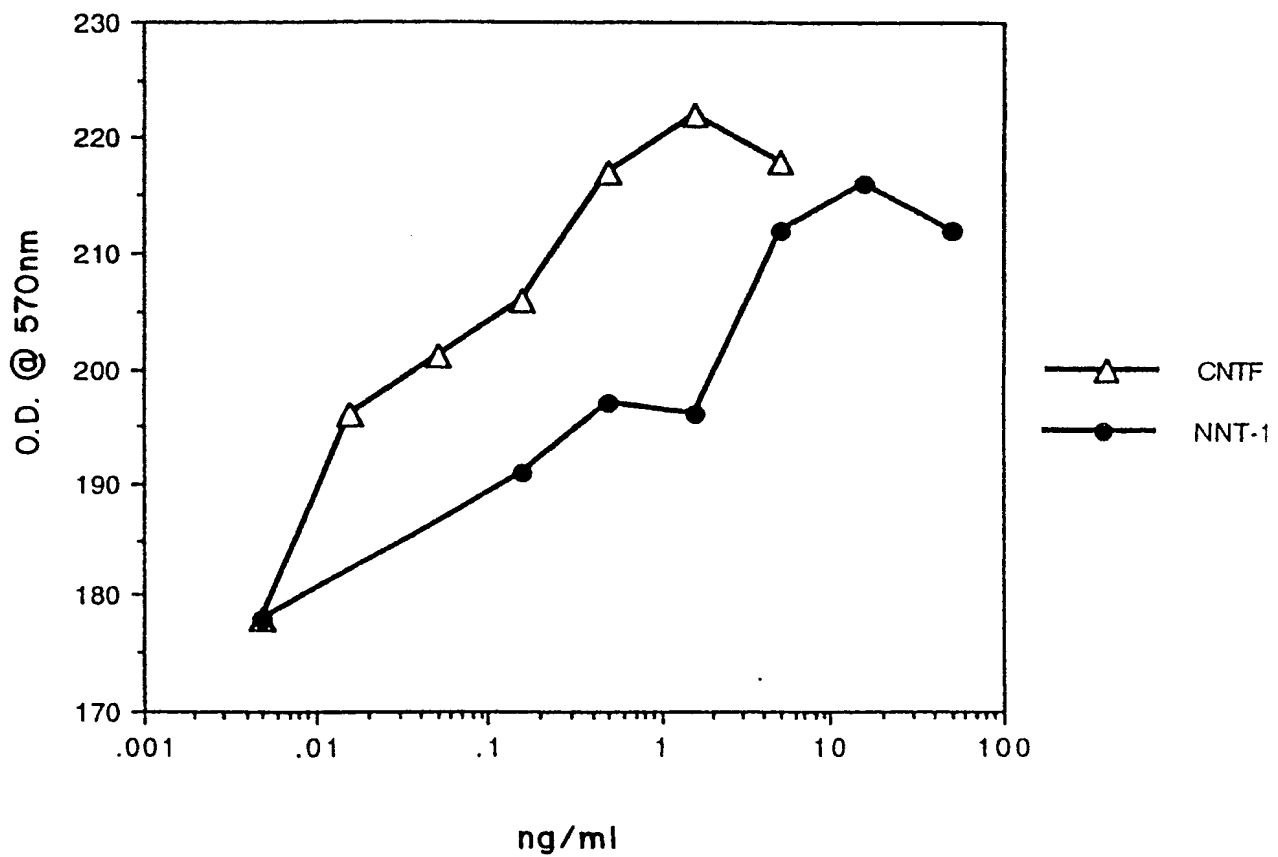
## FIG.6A

	201		250
NNT-1	PQPLPGTEPT WTPGPAHSDF LQKMDDFWLL KELQTLWRS AKDFNR..LK		
Il-11	PPPDPPAPPL APPSSAWGGI ...RAAHAIL GGLHLTLDWA VRGLLL..LK		
Il-6	TPDPTTNASL LTKLQAQNQW LQDMTTHLIL RSFKEFLQSS LRALRQ..M*		
GCSF	ALQPTQGA.. MPAFASAFQR RAG..GVLVA SHLQSFLEVS YRVLRH..LA		
Cardiotrophin	GPRAEPPAAT ASAASATGVF PAKVLGLRVC GLYREWLSRT EGDLGQ..LL		
CNTF	IPRNEADGMP INVGDG.GLF EKKLWGLKVL QELSQWTVRS IHDLRF..IS		
Oncostatin	TKAGRGASQP PTPTPASDAF QRKLEGCRFL HGYHRFMHSV GRVFSK..WG		
LIF	YHVGHVDTVY GPDTSKDV F QKKKLGCQLL GKYKQIIAVL AQAF*		
	251		300
NNT-1	KKMQPPAAAV TLHLGAHGF* .....		
Il-11	TRL*..... .....		
Il-6			
GCSF	QP*..... .....		
Cardiotrophin	.PGGSA*		
CNTF	.SHQTGIPAR GSHYIANNKK M*.. .....		
Oncostatin	ESPNRSRRHS PHQALRKGVR RTRPSRKGKR LMTRGQLPR*.. .....		
LIF			

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FIG. 7

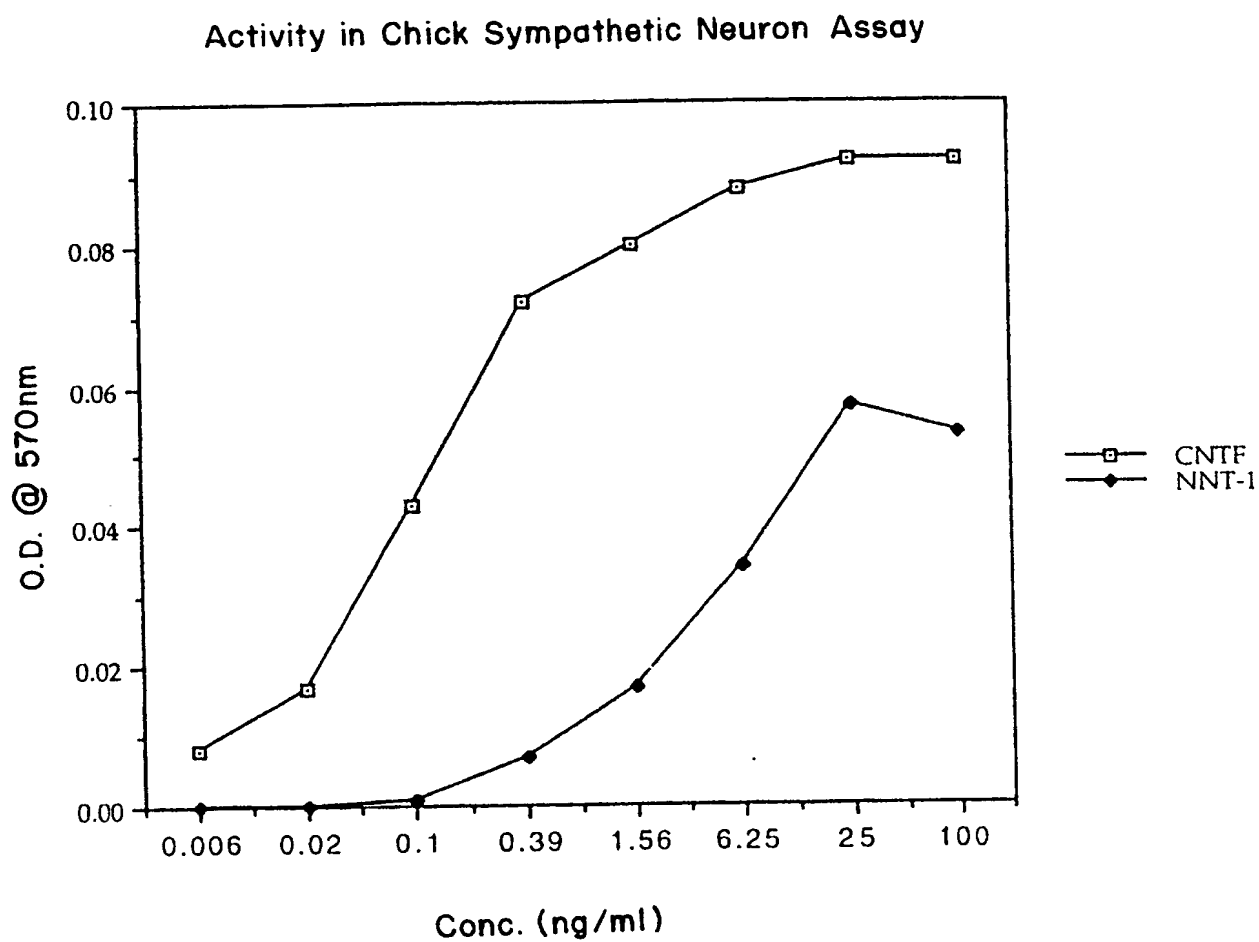
## Activity in Chick Motor Neuron Assay



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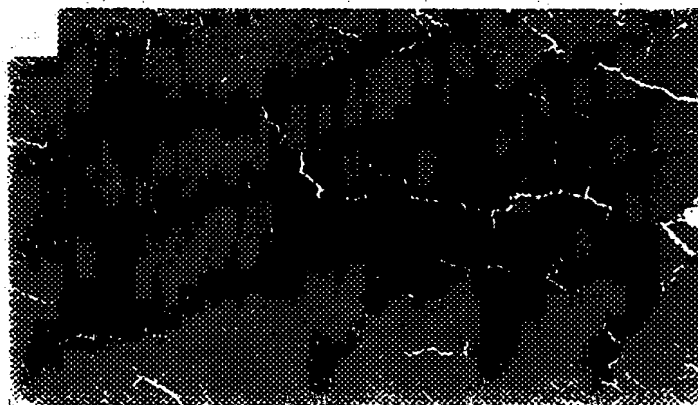
FIG.8



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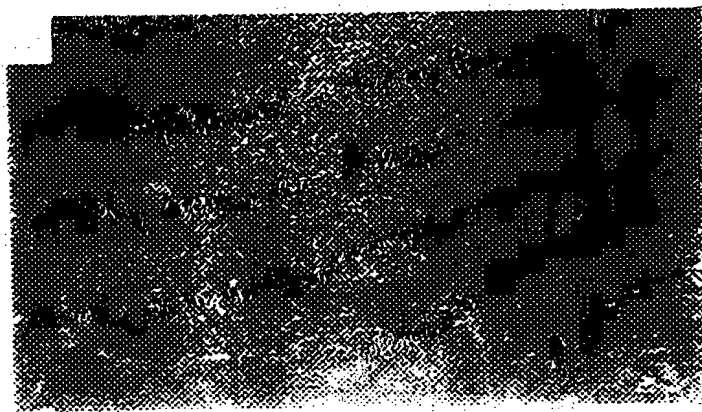
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FIG. 9



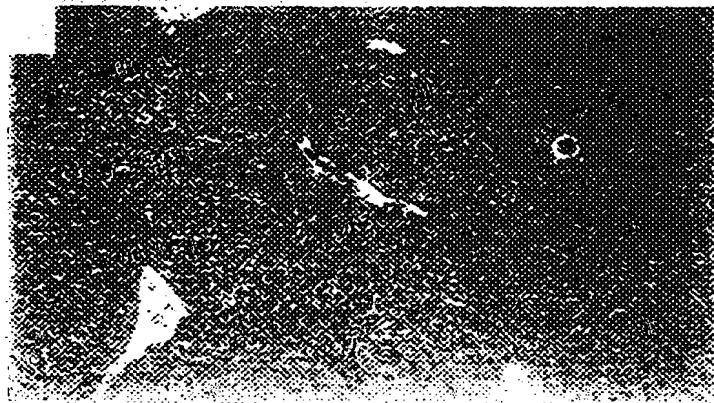
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FIG. 10



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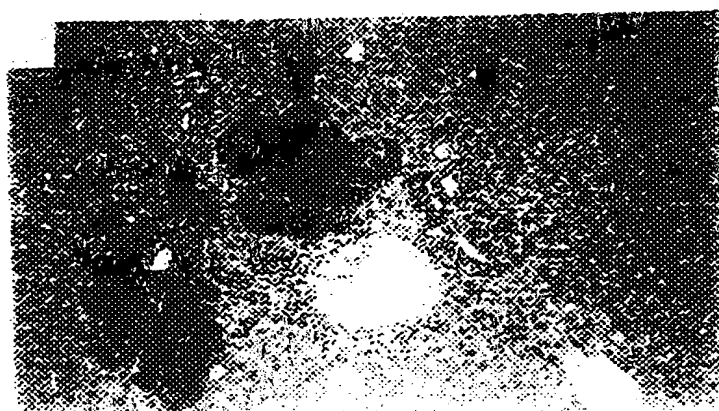
FIG. 11





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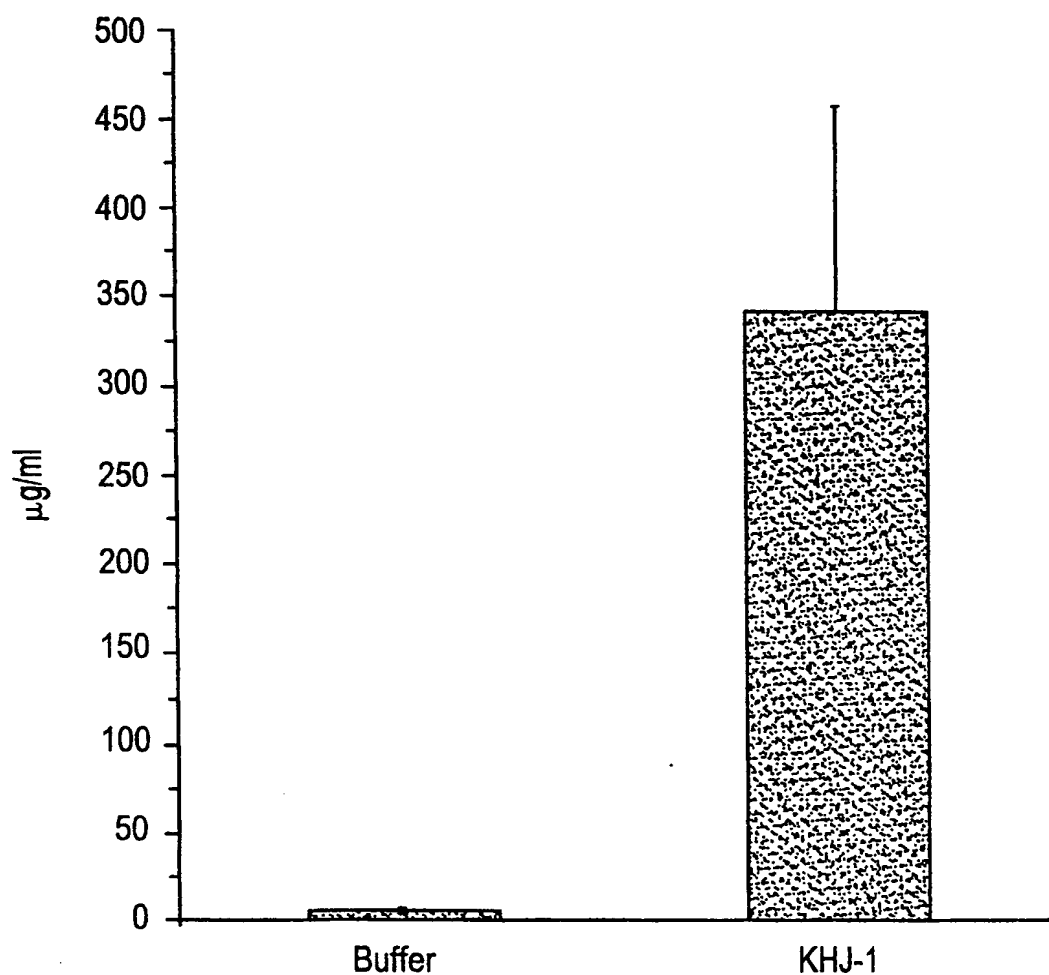
FIG.12



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## FIG. 13

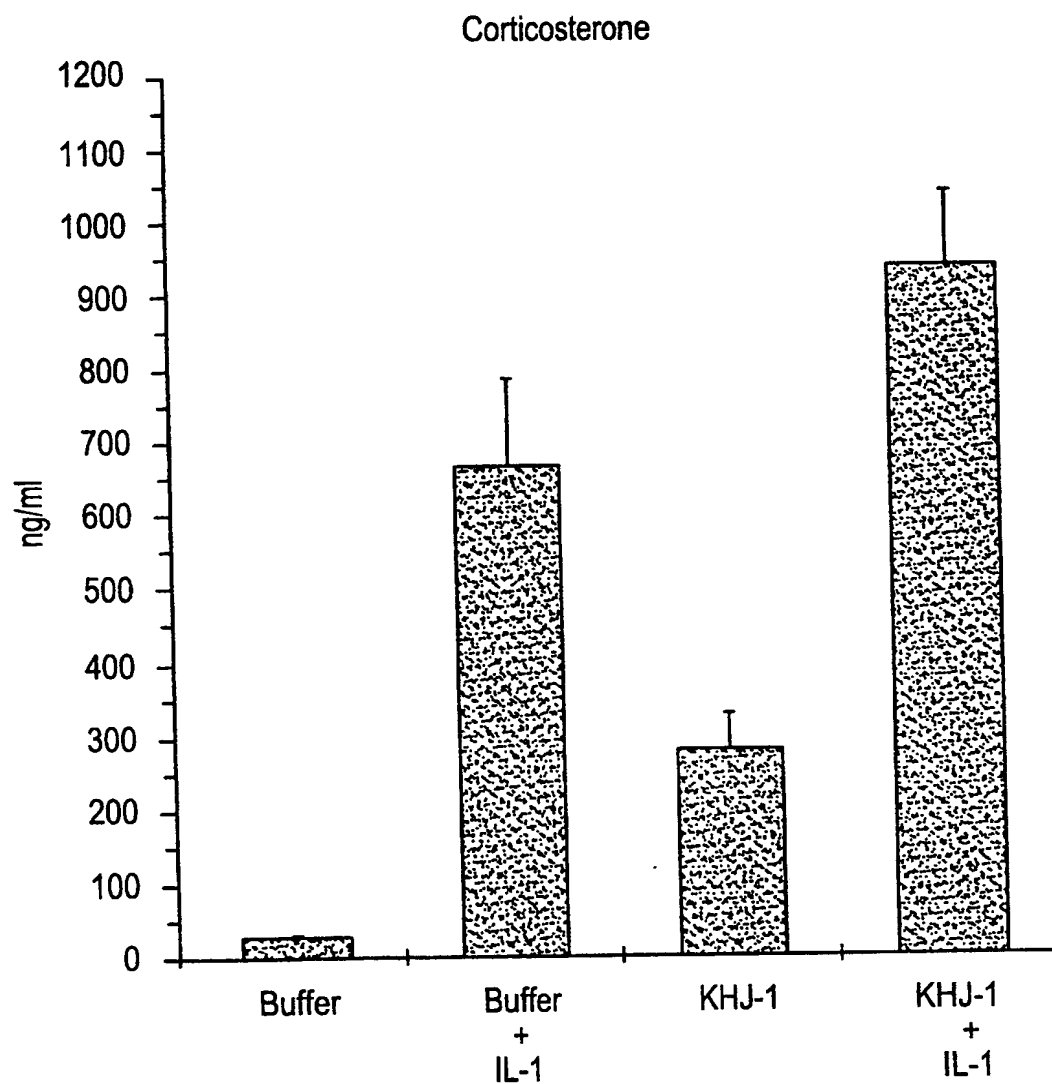
Serum SAA



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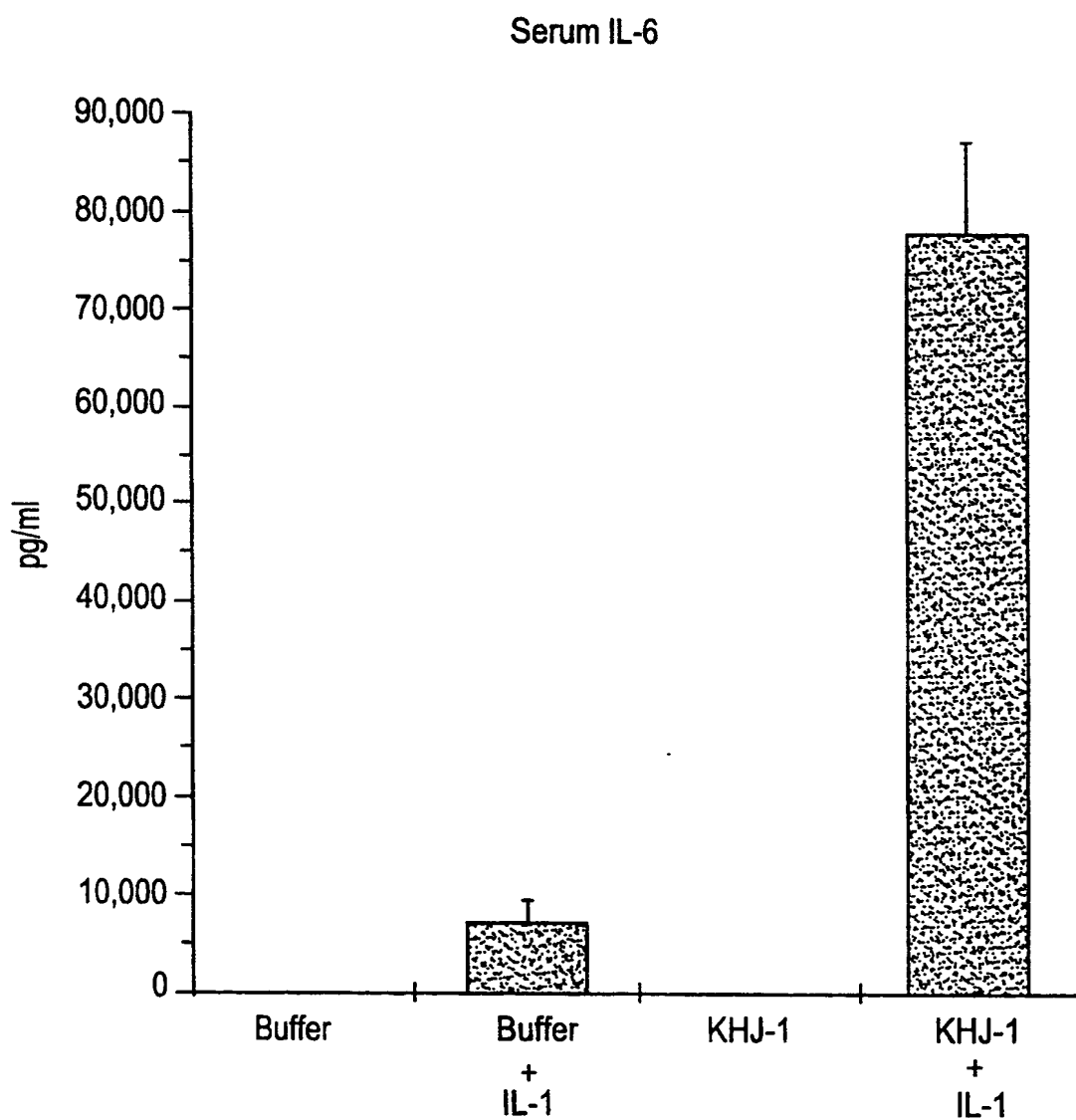
FIG. 14



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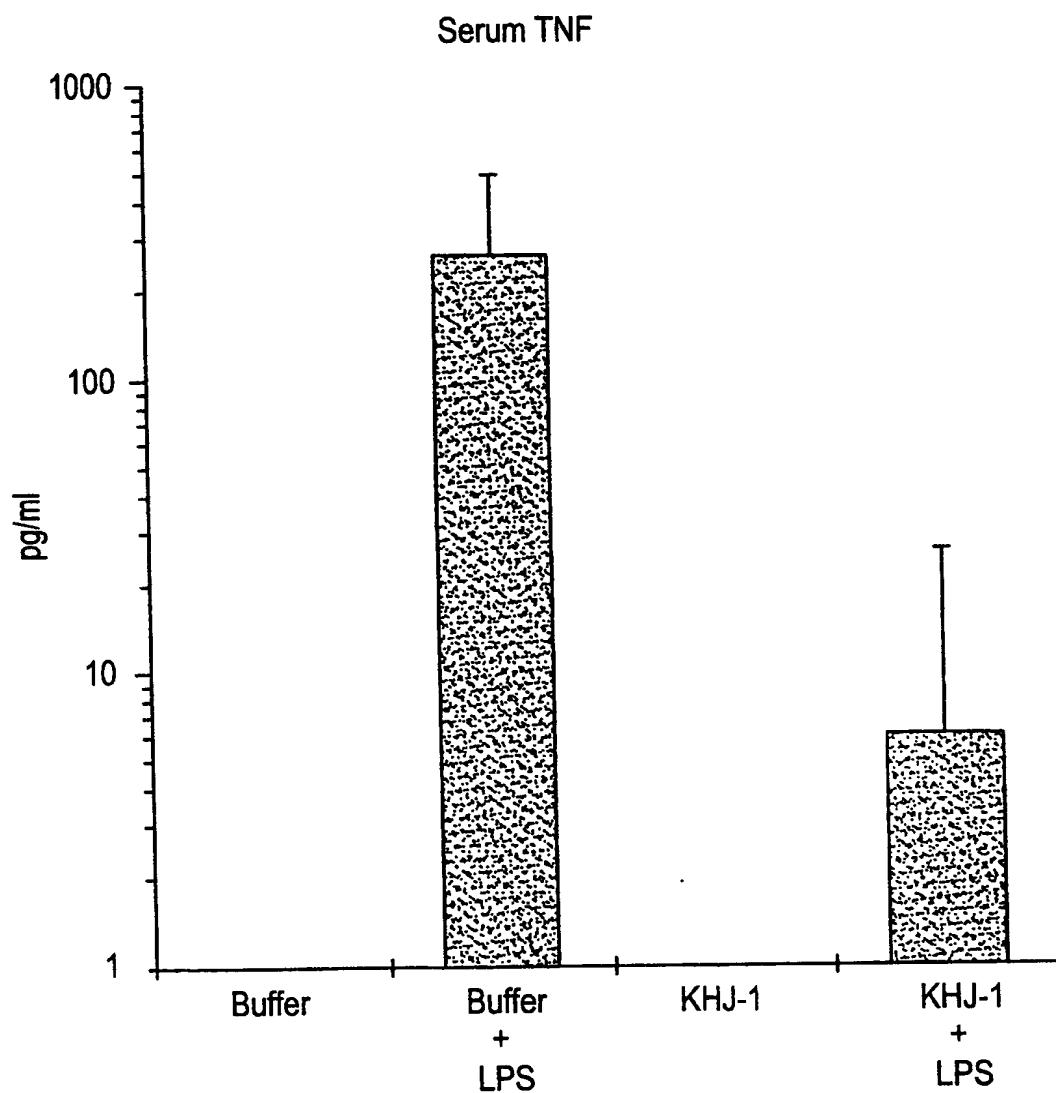
FIG. 15



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FIG. 16



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FIG. 17A

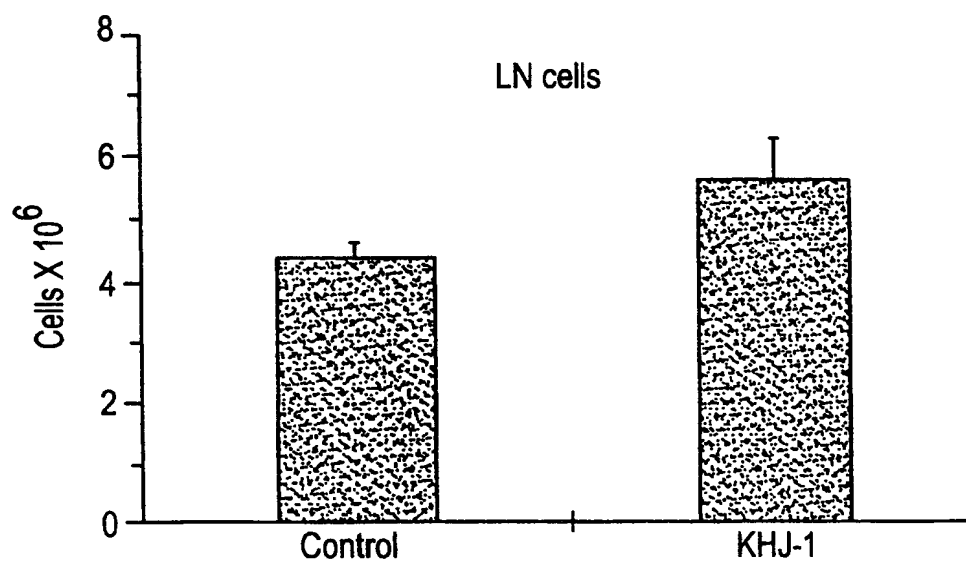
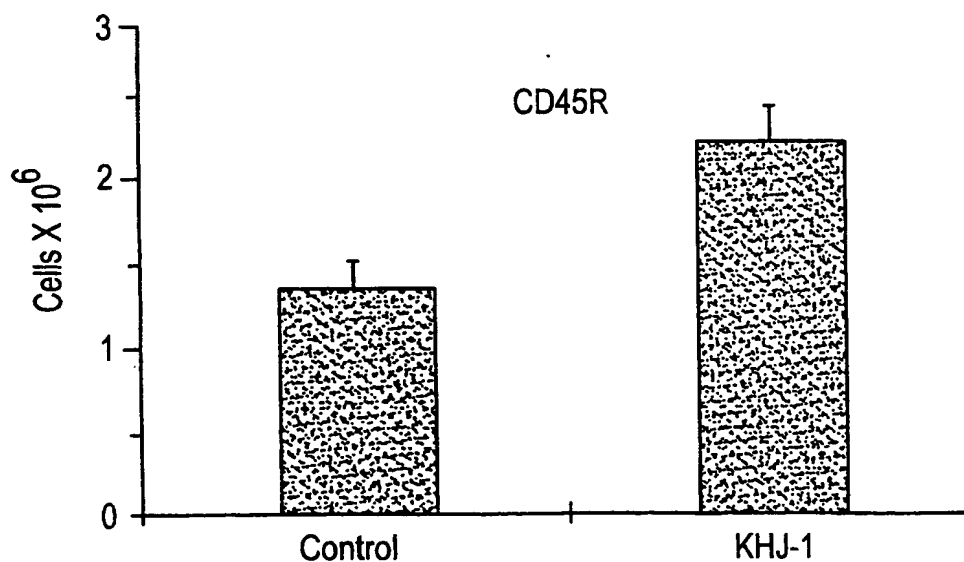


FIG. 17B



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# INTERNATIONAL SEARCH REPORT

Intern 1al Application No

PCT/US 98/02363

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/18 C07K14/475 C07K16/22 C12N1/21 A61K38/18  
A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HUMPEL C ET AL: "Monitoring release of neurotrophic activity in the brains of awake rats." SCIENCE, JUL 28 1995, 269 (5223) P552-4, XP002068930 cited in the application see the whole document ---	1,2,10, 11
A	EMBLdatabase Accession number AA015243 03-AUG-1996 (Rel. 48, Created) Marra m et al. XP002068940 see the whole document --- -/-	1,2,10, 11

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

22 June 1998

Date of mailing of the international search report

09.07.98

Name and mailing address of the ISA

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Authorized officer

Gurdjian, D

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/02363

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BENIGNI, FABIO ET AL: "Six different cytokines that share GP130 as a receptor subunit, induce serum amyloid A and potentiate the induction of interleukin-6 and the activation of the hypothalamus-pituitary-adrenal axis by interleukin-1"</p> <p>BLOOD (1996), 87(5), 1851-4 CODEN: BLOOAW;ISSN: 0006-4971, XP002068931 see the whole document</p> <p>-----</p>	<p>1,2,10, 11</p>



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 98/02363

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 18-23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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